STUDIES ON THE INTERACTIONS OF MULTICOMPONENT CARBON SOURCES IN DISCONTINUOUS AND CONTINUOUS FLOW ACTIVATED SLUDGE SYSTEMS

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CHAPTER I

INTRODUCTION

A. Nature and Importance of the Problem

Currently, aerobic biological methods of waste treatment seem to provide the most economic and most widely used approaches for removing soluble and colloidal organic materials from municipal and industrial waste waters. The activated sludge process is a prominent example of biological treatment. Biological treatment of waste water may be said to be the major industrial "fermentation" process employing continuously grown heterogeneous microbial populations as well as heterogeneous carbon sources. It is therefore a biological system characterized by great complexity rather than simplicity.

It is well known that most biological treatment processes are not revenue-producing operations; however, their impact upon any nation's economy is beyond measure. Recently, the public call for action concerning pollution control and maintenance of rivers and streams in suitable condition for recreation and sources of municipal and industrial water supplies has become one of tremendous urgency. All news media frequently expose the nature of pollution problems to the public with warnings of the consequences to be suffered if pollution is not abated. Clearly, the costly task of cleaning up our water will be a major concern of the decade ahead. It should be equally clear, in the

light of past experience, that research and development of the biological treatment processes will be one of the principal efforts in this decade and beyond.

Over the years many biological treatment processes have been developed, designed, and operated by engineers with little or no knowledge of the requisite biological disciplines. This situation surely will not suffice in the future, since greater control of the aqueous environment will be required, and this engineering control of biological processes cannot be accomplished without knowledge of the biological factors supposedly being subjected to control. The present research represents an attempt by a bioenvironmental engineer to give equally-weighted attention to both the usual engineering factors and the biological factors.

One of the areas about which little has been determined centers around the mixed substrate nature of most waste waters which are subject to biological treatment. The presence of some carbon sources may help or hinder the removal of others. It is important to seek understanding of possible substrate interactions which occur among the various organic compounds present, and to determine the extent and significance of such interactions in heterogeneous or natural populations.

B. Purposes of This Study

The primary purpose of this study was to evaluate substrate interactions and removal patterns for various carbon sources in synthetic waste medium of known composition. Three- and four-carbon source systems were used to investigate the biological responses of naturally selected microbial populations under both batch and continuous flow conditions. The aim of these studies was to provide useful information

concerning kinetics and mechanisms of substrate removal, particularly with respect to minimum aeration tank detention times which can be employed with minimal leakage of carbon sources from the reactor. Furthermore, when completely analyzed and interpreted in the light of knowledge concerning various whole wastes, the aim of these studies was to provide possible insights into more reliable design procedures than are presently employed in the field.

Since biological waste water treatment processes are even more heterogeneous with respect to the biological population than the carbon sources they contain, some attention was given to microbiological interactions which occur in these systems, i.e., changes in microbial predominance.

Also, because it has been found that during metabolism of a sole source of carbon metabolic products can accumulate in the medium thus creating a multicomponent substrate system, some experimentation to assess the degree of production and utilization of such carbon sources was conducted under two cultural conditions (growing and nonproliferating). In these experiments, aeration was carried out for an extended period of time because it was desirable to assess the availability of the sludge mass as a substrate after the external carbon sources had been removed.

CHAPTER II

LITERATURE REVIEW

A. Metabolic Control Mechanisms

It is characteristic of microorganisms that all of their metabolic activities are highly ordered, and this implies the existence of efficient regulatory mechanisms in these biological systems. These mechanisms are a natural result of evolution, because they permit more efficient energy utilization for living systems. Five metabolic control mechanisms operative at either the genetic level or the enzymatic level are known and have been characterized. Enzyme repression and feedback inhibition are operative in biosynthetic (anabolic) pathways, whereas enzyme induction, metabolic repression (glucose effect), and metabolic inhibition operate in degradative (catabolic) pathways. A diagrammatic summary of control mechanisms shown by Gaudy and coworkers to be applicable to heterogeneous populations was reproduced from a research seminar (Gaudy, personal communication) in the author's M. S. thesis (1). Control of amphibolic pathways was recently reviewed by Sanwal (2).

Theoretical concepts pertaining to these mechanisms have been reviewed by Grady (3). Hence it is not necessary to review all of these well-known mechanisms in this text.

In addition to the above mechanisms, permease competition or

interference with the transport of a substrate molecule into a bacterial cell by another substrate can cause substrate interactions. The existence of this selective bacterial transport system has been reviewed by Heidman (4).

Although much is now known concerning metabolic control mechanisms which bring about the orderly progress of carbon and energy flow and the mode of removal of substrate, much basic research is needed to delineate the mechanisms further and to define various metabolites which may govern the action of these mechanisms. From the standpoint of understanding and perhaps eventually controlling the cause of purification of polluted water in natural ecosystems, it is the substrate interactions, i.e., the end result of the operation of the control mechanisms, which are of more critical importance to the bioenvironmental engineering-science field.

It was an observation of an end result of the operation of a control mechanism, i.e., "diauxic growth," which fostered the development of avenues of research leading to the present basic areas of bacterial genetics and control mechanisms.

B. Substrate Interactions in Biological Systems

In experiments with *Escherichia coli* and *Bacillus subtilis*, Monod (5) observed that with some mixtures of compounds (glucose and aliphatic polyhydric alcohols) the growth curves exhibited two successive growth cycles which were separated by a lag. There is now little doubt that the diphasic growth curves were caused by sequential removal of glucose and polyhydric alcohol, and that this sequential growth was the manifestation of the effects of repression of enzyme synthesis. It was enzyme induction and repression which interested Gaudy in his studies on

sequential substrate removal by heterogeneous populations (6). His experiments demonstrated that a diphasic growth of heterogeneous populations resulted from sequential removal of glucose and sorbitol; he measured substrate removal as well as growth.

Springing from these early findings (Monod in pure cultures, and Gaudy in heterogeneous populations), a new and vital area of bioenvironmental research has been initiated. Since bioenvironmental engineers usually work with heterogeneous or natural populations of microorganisms, this literature review is concerned primarily with the expanding research on control mechansims and substrate interactions as found in natural microbial ecosystems.

a. Batch Systems

After Gaudy's initial experimentation, an active research program concerning sequential substrate removal in heterogeneous population systems was begun by Gaudy and his associates (7)(8)(9)(10)(11)(12)(13). Brief reviews pertaining to this publication series have already been given by Grady (3), Yu (14), Heidman (4), and Kiravanich (15). The results of these studies have shown that metabolic control mechanisms can play a determinative role in biological treatment systems. Their operation can lead to discontinuities in kinetics of waste water purification under qualitative shock loading conditions in multicomponent carbon source media.

In order to check Gaudy's reports of sequential substrate removal, Prakasam and Dondero conducted two investigations (16)(17) in which a sorbitol-acclimated sludge and a sludge taken from an activated sludge plant were employed. Using the "young cell" culturing technique (identical to that used by Gaudy) and radioactively labelled sorbitol, they

confirmed independently the sequential removal of glucose and sorbitol by a sorbitol-acclimated population. However, they concluded that concurrent removal of glucose and sorbitol occurred in the experiments with activated sludge taken directly from a sewage treatment plant. They felt that the concurrent removal occurred because the activated sludge had a greater variety of microbial species than did the adapted sludge. As pointed out by Grady (3), they did not present any data for glucose utilization in the mixed substrate; also, their activated sludge sample can be classified as an "old cell" suspension of the type which had been shown by Gaudy, et al. to yield concurrent removal.

In an attempt to confirm the "relevance" of Gaudy's finding, Stumm-Zolliner (18)(19) also has studied metabolic control mechanisms in mixed microorganism systems. She extended the range of experimental conditions and employed various substrate systems. Her results solidly confirmed that substrate interference is commonly observed in heterogeneous populations growing on multicomponent substrates.

However, like Prakasam and Dondero, she was quite concerned over the loss of diversity of microbial species which might occur during acclimation to a specific compound. Her concern in this regard has already been criticized by Grady (3). Actually, the term "acclimation" used by the sanitary engineer is merely the progressive selection of microorganisms best suited to utilize the particular waste under the conditions prevailing in the treatment plant. There is little doubt that this selected microbial population will offer an ideal test condition for the study of substrate interference. Therefore, acclimation should be regarded as an advantage for the study, not a disadvantage, i.e., preacclimation offers a more severe test of the concept

than does use of a nonacclimated population.

Recently, Grady, et al. (20), and Grady and Gaudy (21) reported results of further experiments using glucose-lysine (20), fructose-lysine (21), and ribose-lysine systems (21). In these studies they harvested lysine-grown cells from a batch-operated fermentor and grew these cells through one transfer on lysine, glucose, or a mixture of lysine and glucose. They compared the substrate removal rates and enzymatic capabilities of the cells in lysine medium, and concluded that the synthesis of lysine-degrading enzymes was subject to catabolic repression by glucose and fructose, whereas ribose caused a slight increase in the synthesis of lysine enzymes. They also found that glucose played only a minor role in inhibition of the activity of the preformed enzymes, whereas fructose did not inhibit enzyme activity. Since the research involved essentially a "whole cell assay" in which the rate of synthesis lysine-metabolizing enzyme(s) was compared with rate of synthesis of total protein, it was one of the most penetrating studies of repression and inhibition phenomena ever accomplished with heterogeneous populations.

Numerous investigations have studied substrate interactions in pure culture systems operated under batch conditions. However, only one of the latest reports, which is related directly to the results to be presented later, will be reviewed briefly here.

McGinnis and Paigen (22) studied a young culture of Escherichia coli pre-grown on a variety of radioactive sugars, including galactose, lactose, maltose, mannose, L-arabinose, xylose, and glycerol. They found that only glucose and, to a lesser extent, glucose-6-phosphate, were capable of inhibiting the utilization of all of the radioactive

substrates. Their experiments with mutants blocked in subsequent steps of galactose and lactose metabolism demonstrated that the inhibition occurred prior to the formation of the first metabolic product by glucose. From these results they concluded that Escherichia coli has a general regulatory mechanism, termed "catabolite inhibition," which controls the activity of early reactions in carbohydrate metabolism, allowing certain substrates to be utilized preferentially. This work substantiates the conclusions of Gaudy and his associates concerning the existence of a general "feedback inhibition" in a catabolic pathway. However, they pointed out that the inhibition is not necessarily brought about by metabolic intermediates.

b. Continuous Flow Systems

During the growth of a microorganism in a batch system, the growth rate changes during the course of substrate utilization and the chemical composition of the medium varies as a result of the metabolism of the organisms. Under such circumstances it is difficult to assess all of the factors which control the physiological state of the cells. On the other hand, a continuous flow system offers some advantages in minimizing the effect of environmental factors, provided a "steady-state" condition can be attained; the steady-state can then be altered and differences in the behavior of the system in the steady and transient conditions can shed light on the operation of various metabolic control mechanisms.

Unfortunately, information on studies of control mechanisms in continuous heterogeneous cultures is scarce. The first published research article of this type was the paper by Komolorit and Gaudy (23). They reported the transient responses of heterogeneous populations to

shock loadings of glucose and sorbitol for cells previously growing on sorbitol.

In order to determine whether their findings in the batch systems (20)(21) were also manifested under continuous flow conditions, Grady and Gaudy (24) operated continuous flow reactors on L-lysine under various conditions, and then changed the influent substrate to a mixture of L-lysine and glucose, L-lysine and fructose, or L-lysine and ribose. They observed that in all cases glucose and fructose caused a significant repression of the synthesis of lysine-degrading enzymes, whereas ribose caused an initial increase in lysine enzymatic capability followed by a slight repression when ribose degradation started. They also observed that in a carbon-limited system the faster the flow rate, the greater was the repression. This series of experiments led them to conclude that the degree of repression depended upon the carbohydrate used, and that the relative effectiveness of the three sugars to repress L-lysine was the same in both batch and continuous flow systems; i.e., glucose > fructose > ribose.

Kiravanich (15) has recently completed a study in which multicomponent substrate systems (two- and three-carbon sources) were employed in both batch and continuous flow experiments. The aim of his studies was to determine if the dilution rate, D, had an effect on sequential substrate leakage in the effluent. From the results of the batch and continuous flow studies using glucose-glycerol-galactose and glucose-glycerol combined carbon sources, he concluded that increasing flow rate ($D = 1/24$ to $1/3$ hr^{-1}) in multicomponent carbon systems led to leakage of the substrates in the same order as that observed in batch systems. He also introduced a quantitative shock loading to a two-component

carbon source system. In this study the dilution rate was maintained at 1/8 hr^{-1} while increasing the feed concentration from 500 mg/l to 4000 mg/l (equal portions of glucose and glycerol). From the results he observed that increasing severity of quantitative shock loading in a glucose-glycerol system led to significant leakage of glycerol. When glucose feed was held constant and glycerol concentration was increased, he found a less severe leakage of glycerol. These results indicate that the severity of glycerol blockage was associated with the ratio of glucose to glycerol in the feed.

Other than the research by Gaudy and his co-workers, the only other report dealing with heterogeneous continuous cultures on mixed substrates in which analyses for specific substrates were made is that of Chain and Mateles (25). They operated a continuous flow reactor fed a mixture of glucose and lactose or glucose and butyrate along with a small amount of yeast extract. They found that glucose was used in preference to the other carbon sources, and the utilization of the secondary carbon source was greatly reduced at high dilution rates. There was a discontinuity in butyrate utilization between D = 0.2 and 0.4 hr^{-1} but there was no discontinuity in the release of lactose into the effluent. They also conducted similar research using pure cultures in a glucose-fructose system (26). With Escherichia coli at low dilution rates, both sugars were utilized completely, while at high flow rates much of the fructose was released into the effluent. With Pseudomonas fluorescens, the growth rate at which fructose utilization was impaired was particularly low; more than 200 mg/l of the 500 mg/l feed remained unutilized at D = 0.3 hr^{-1}. The response of Saccharomyces cerevisiae was different from that of the bacteria. In this yeast, less fructose

than glucose was removed at all growth rates, but the utilization of these two sugars was "competitive" throughout the experiment, i.e., the presence of glucose did not prevent fructose removal.

C. Relative Potential for Substrate Interference and Substrate Removal Patterns

Mandelstam's studies of "metabolite repression" (27) have shown that the rate of utilization of a carbon source is of primary importance in establishment of repression. In an article which reported on studies in which glucose and glycerol were mixed carbon sources, Zwaig and Lin (28) indicated that the blockage of glycerol was due to the inhibition of glycerol kinase by accumulated fructose-1,6-diphosphate. From his research data, Yu (14) concluded that when growth on one of the compounds as a sole carbon source proceeds at a rate significantly faster than growth on another compound, or when growth on one compound leads to the accumulation of a significantly higher level of metabolic intermediates, the presence of that compound in a combined substrate system will tend to retard the removal of the other substrate.

Based on these previous findings, the relative potential a substrate may possess for interference with the utilization of another substrate can be characterized by 1) growth rate in the single substrate system, 2) production of metabolic intermediates, and 3) acclimation period (if required to initiate metabolism). If compound "A" has a higher potential than compound "B" as shown in the control systems, compound "A" will interfere with the utilization of compound "B" when these compounds are combined. The order of removal in the mixture most likely will be compound "A" > compound "B."

In accordance with the preceding review pertaining to substrate

interactions and the results of Su's studies (1), three common patterns of substrate utilization for two-component carbon systems can be distinguished: 1) the population may consume two compounds simultaneously (the rates of removal of each may or may not be affected by the presence of the other); 2) first one compound may be used and then the next, because of inhibition of the activity of the enzymes that promote the use of the second compound ("B"); 3) first one compound may be used and then the next, because of repression of the formation of enzymes which initiate the utilization of the second ("B") compound.

Although at the present time there is no obvious or easy way to reduce such knowledge for immediate use by today's practicing engineer, knowledge of the metabolic patterns and mode of removal for mixed substrates has obvious future practical significance regarding joint treatment of various industrial wastes and/or industrial wastes and municipal wastes. As the problems and demands for water reuse become more acute and the need for understanding the behavior of natural microbial systems becomes more and more apparent to the engineering and scientific community, the utility and practical significance of these types of studies will also become more apparent.

D. Operational Conditions and Substrate Interactions

Numerous investigations have been conducted to determine operational conditions under which substrate interference may or may not occur and the extent to which operational conditions may or may not be used to gain some sort of control over such occurrences.

a. Cell Age

A series of studies have been undertaken in the bioenvironmental

engineering laboratories at Oklahoma State University to determine the effect of "cell age" on substrate removal in a multicomponent carbon source system. In 1963, Gaudy and his associates (7) showed that older cells acclimated to sorbitol tended to remove glucose and sorbitol concurrently, whereas younger populations were found to remove the glucose from the combined system prior to utilization of the sorbitol. The concern of Prakasam and Dondero (16)(17) and Stumm-Zollinger (18)(19) over the degree of heterogeneity of the populations actually can be explained in the light of Gaudy and his associates' findings of the sludge age effect. The municipal activated sludge used by the other investigators cited above could be rightly characterized by the term "old cells." Their results apparently indicate that increased ageing of the sludge (highly flocculated cells), as opposed to young dispersed cells grown up from small inocula, tended to decrease manifestation of sequential substrate removal. However, Yu (14) found that it was impossible to make generalized statements concerning the effect of cell age, as determined by retention time in a chemostat, on the substrate removal patterns in a glucose-glycerol medium. He observed a general tendency for decreased manifestation of sequential removal for cells originally grown at lower growth rates. However, he also observed concurrent removal to occur for cells originally grown at faster growth rates.

Heidman (4) ran batch studies and found that continued operation of the batch unit (continued ageing) did not significantly increase the degree of concurrency of substrate removal. However, young cells (one-day operation) exhibited a much higher degree of substrate interference than an older population (e.g., eight days of operation). In a system consisting of galactose and glucose, galactose was removed at

approximately two-tenths the rate it was removed in the control unit when the young cells were employed, whereas for the older cells the ratio was above 0.5. Thus it would appear that young cell systems may be expected to exhibit a greater degree of substrate interference. However, it is noted that in the galactose-glucose system, glucose did interfere with removal of galactose at all "cell ages" examined; after the "8-day" experiment, the ratio of galactose removal rates in the combined and control systems ranged from 0.4 to 0.6. While the effect does appear to be somewhat less manifest for "older" populations, it is one which cannot be overlooked regardless of the age of the population.

b. Growth-limiting Nutrients

Work previously reviewed has shown that substrate interference due to control mechanisms can occur in heterogeneous populations under carbon-limiting conditions. It should be noted that most of those studies were accomplished in high-energy systems and were primarily applicable to biological waste treatment processes. In 1965, Bhatla and Gaudy (29) extended the investigations of glucose-sorbitol interactions to more dilute (low energy) systems which exist in natural waters. They observed that sequential metabolism of these substrates (5 mg/l each) resulted in a diphasic curve of accumulated oxygen uptake wherein the two phases were separated by a discernible plateau. Thus it would appear that glucose catabolites caused repression of the synthesis of sorbitol-degrading enzymes even at low concentrations.

Gaudy, et al. (7) reported that the sequential removal of glucose and sorbitol also occurred under nitrogen-deficient conditions. Later work by Grady, Gaudy and Gaudy (20) showed that the absence of ammonia nitrogen in a glucose-lysine system did not overcome the effect of

glucose on lysine metabolism.

Grady and Gaudy (24) also extended their studies of glucose-lysine interactions in continuous systems to magnesium-limited conditions. They showed that at lower flow rates, repression of lysine by glucose was greater than at high flow rates. An opposite effect of dilution rate was observed in a carbon-limited reactor by these workers. Thus it would appear that the degree of lysine repression due to glucose varied with both dilution rate and limiting nutrient.

It should also be mentioned that ability of one substrate to interfere with metabolism of another is not a unique characteristic of glucose. From the results of studies in which a butyrate-phenol system was employed, Chain (30) concluded that even in the presence of a relatively poor carbon source, such as butyrate, phenol was subject to strong catabolite repression in a continuous flow carbon-limited (pure culture) system.

c. Salt Concentrations

In the previous studies it has been shown that sequential substrate removal can occur when two exogenous substrates are added (7) or when the cells produce exogenous substrate during metabolism of an original exogenous compound used as a sole carbon source (31). Another possible mode of occurrence of sequential substrate removal was observed by Kincannon, Gaudy, and Gaudy (32). In their study, cells grown on glucose in high salt medium (45,000 mg/l) were subjected to a shock loading of salt-free medium. They observed sequential removal of the original exogenous carbon source (glucose) and cellular material released as a result of the severe osmotic shock. This finding adds additional evidence for the occurrence of sequential substrate removal in heterogeneous

populations and demonstrates another operational condition under which it occurs.

The above brief history of past work shows that the variety of environmental changes which can be examined is large, and that continued research into the mode of substrate removal in multicomponent media seems warranted. It was pointed out by Gaudy some years ago (33) that one of the important modes of response to changes in the nature of the carbon sources in waste (qualitative shock loads) which had to be considered was changes in predominance of species. An activated sludge represents a highly complicated ecological system, and such a system is subject to an ever-changing predominance of microbial species. Ecological studies of microbial interactions are needed in order to discern various trends and effects.

E. Microbial Ecology and Microbial Interactions in Biological Systems

Microbial ecology is the study of interactions of microorganisms and environments. The ecological approach to research on activated sludge systems emphasizes the influence the biological population exerts on the performance of the treatment processes. One of the desirable goals of the "sanitary" engineer is to determine which organisms play a beneficial role in activated sludge systems, and to encourage the growth of the desired organisms and discourage or eliminate the growth of unneeded or nuisance organisms. Two main avenues of approach may be followed for research on microbial interactions: 1) using a natural population, environmental (experimental) conditions may be varied, and observations of changes in the population made; 2) species may be isolated, and specific organisms studied in pure cultures (34).

Gaudy and his associates have already accomplished much work using natural populations (1)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(20)(21)(23)(24)(29)(31)(32)(33), and additional work is in progress. Also in line with the second avenue of approach, they have already completed significant investigations (35)(36)(37)(4). In one of these studies, cultures isolated from municipal sewage have been studied in mixed cultures (36).

In a general sense, the phrase "survival of the fittest" aptly characterizes natural populations in regard to the environment in an activated sludge plant. According to various roles they have in the system, organisms can be classified as: floc-forming organisms, saprophytes, predators, and nuisance organisms (34). The organisms involved in each of these roles may include a wide spectrum of microbial populations, such as bacteria, fungi, protozoa, or invertebrates.

In general, the following ecological factors (or environmental factors) are believed to determine which organisms will be present in heterogeneous populations: 1) weather--including temperature, light, etc., 2) nutrition--types of food available; 3) oxygen supply--anaerobic or aerobic environment; 4) pH; 5) toxicity--including the presence and the level of toxic materials; 6) ecological niche--the habitat in which they live (34)(38). Certain environmental factors such as temperature, nutrition, oxygen supply, pH, and toxicity can be held constant or varied in a known and controlled fashion in the laboratory, the variety of combined environmental conditions which can be studied is great, and much work of this type will be needed in order to assess cumulative and combined effects on species predominance.

Pipes (34) has characterized the microbial interactions into three

distinct categories: competition, predation, and synergism. He also pointed out that microbial population changeover in heterogeneous populations is attributed mainly to: 1) competition (faster-growing species over slower-growing ones); 2) predation; 3) stimulation by nutrilites, vitamins, or small quantities of antibiotics. In addition to the interactions between the organisms, mutation undoubtedly occurs in continuously-grown heterogeneous cultures. It is understandable that the mixed cultures extant in biological treatment plants exhibit great variety in view of the fact that changes in environmental factors are often encountered. Since each environmental factor is expected to act as an agent in selecting the best-suited organisms, there is little doubt that the heterogeneous populations in biological treatment systems are truly dynamic systems. It is conceivable that under the conditions prevailing in the processes, only those organisms which are able to grow faster than the other species ultimately will predominate.

Because of the complexity of species interaction and the experimental problems involved, studies on population dynamics are carried out using simplified model systems. Various two- and three-culture continuous flow systems have been investigated in different laboratories (39)(40)(41) to determine various types of interactions in terms of dynamic population responses under various environmental conditions. Bustamante (36) conducted batch and continuous flow studies using combinations of two or three microorganisms isolated from sewage. He detected that an interrelationship between Pseudomonas aeruginosa and other organisms benefited the growth of Pseudomonas aeruginosa in the presence or absence of exogenous substrate. He suggested that the soluble pigment produced by Pseudomonas aeruginosa might have induced

lysis, or retarded growth of other cells. He also pointed out that changes in predominance or in relative numbers of bacterial species present in continuous flow systems can be brought about in response to quantitative, qualitative, and hydraulic shock loadings. However, research on microbial interactions in heterogeneous population systems is still in its infancy. Edwards (42) reported that at higher dilution rates (under ambient temperature conditions) mixed culture tends to favor attached organisms because free-swimming organisms are washed out before dividing. Cassell, et al. (43) studied population dynamics in continuous flow systems developed from natural sewage microorganisms using skim milk as substrate. They observed that at various constant detention times all parameters which reflected biological activity fluctuated continuously, and concluded that these fluctations were the results of microbial interactions. They successfully established different predominating populations at high and at low detention times. In some of the continuous flow systems, frequent population changeovers were observed. Thus they pointed out that in a heterogeneous microbial population, detention time was a strong selective agent and more than one population type could predominate. However, they also concluded that when two mixed populations competed and neither had a great selective advantage, the mixed culture would be unstable and might produce relatively large variations in effluent quality. Cassell (44) also reported that over the range of detention times investigated, two distinctly different mixed cultures existed, one at short detention times and another at high detention times. At the intermediate detention times, neither mixed culture could predominate for a long period of time. The occurrence of frequent population changeovers was induced

by the lack of a large selective advantage by either mixed culture. A symbiotic growth between pseudomonads and coliforms was observed by Chain and Mateles (25) in a naturally selected heterogeneous population under both batch and continuous flow conditions. The species of Pseudomonas studied could not grow alone on the glucose-butyrate medium; however, when a natural population was used, these organisms dominated the population in continuous flow systems at high dilution rates as well as in batch systems with high levels of glucose, whereas the coliforms were the predominant organisms in continuous flow systems at low dilution rates and in batch systems with low levels of glucose. It is important to mention that Krishnan and Gaudy (35) also offered some evidence of the symbiotic growth between some species. They found that it is possible to isolate organisms from a laboratory activated sludge system inoculated with sewage which cannot grow alone on the carbon sources in the incoming waste stream.

CHAPTER III

MATERIALS AND METHODS

A. Substrates and Standard Synthetic Wastes

Glucose, sorbitol, glycerol, propionic acid, and butyric acid were reagent grade chemicals obtained from Fisher Scientific Company; galactose, fructose, ribose, and xylose were purchased from Eastman Organic Chemicals; and sucrose was purchased from Nutritional Biochemicals Company. These compounds were chosen because they were known to be present in various waste waters, and reasonably accurate quantitative analyses were available for their determination. Also, a considerable amount of research on the metabolism of these compounds had already been accomplished in either bioenvironmental engineering or microbiological laboratories.

The standard synthetic wastes used as feed medium during these studies consisted of a specific organic carbon source (and combinations thereof) and essential inorganic salts dissolved in distilled water. The inorganic salts used per 1000 mg/l of carbon source were: $(NH_4)_2SO_4$, 500 mg/l; $MgSO_4 \cdot 7H_2O$, 10 mg/l; $FeCl_3 \cdot 6H_2O$, 0.5 mg/l; $MnSO_4 \cdot H_2O$, 10 mg/l; $CaCl_2$, 7.5 mg/l; tap water, 100 ml/l; 10.0 M potassium phosphate buffer at pH 7.0 (KH_2PO_4, 527 mg/l and K_2HPO_4, 1070 mg/l), 10 ml/l. This waste was designed to make the carbon source the limiting nutrient. Whenever the carbon source concentration used was more or

less than 1000 mg/l, the inorganic salts and buffer were proportionally increased or decreased. When 500 mg/l of butyric acid or propionic acid was included as one of the joint substrates, 1830 mg/l or 2440 mg/l additional K_2HPO_4 was added to the standard growth medium. The pH of the synthetic wastes was 6.8 to 7.0.

B. Description of Apparatus and Development of Heterogeneous Microbial Populations

a. Apparatus

The short-term batch experiments were performed in 4-inch diameter test tubes. This reactor, with a nominal volume of 4.0 liters, was completely mixed by aeration of the reaction liquor through a coarse gas dispersion tube.

The apparatus used for continuous flow experiments was a single stage, completely mixed chemostat as shown in Figure 1. The reactor was cylindrical, with an aeration liquor volume of approximately 2.4 liters, had effluent discharge around the entire upper edge, and was submerged in a constant temperature bath (Precision Lo-Temptrol, Precision Scientific Co.) maintained at $25^{o} \pm 0.5^{o}$. Two coarse gas dispersion tubes were used to provide adequate mixing and aeration. Feed was delivered to the reactor by a Milton-Roy Mini-flow pump (Model 4-C-48-R) which was previously calibrated to give the desired flow rate. Suction and delivery lines were made up of tygon tubing with glass junctions, and were cleaned every other day with a Clorox cleaning solution. Dilute-out curves for dilution rates of 1/24 and 1/2 hr^{-1} (Figure 2) confirmed that the reactor was completely mixed with respect to soluble material (substrate).

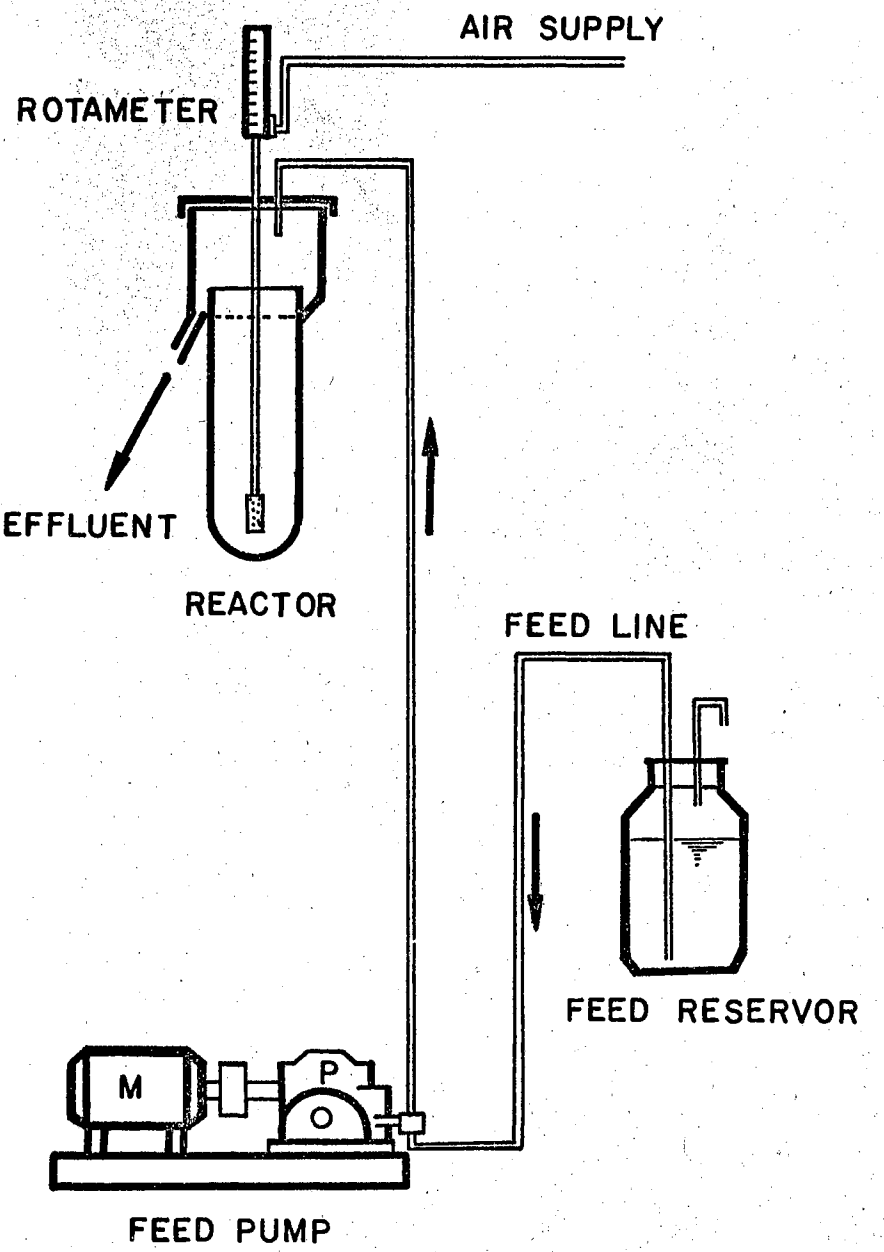

Figure 1 - Schematic representation of a continuous flow reactor apparatus.

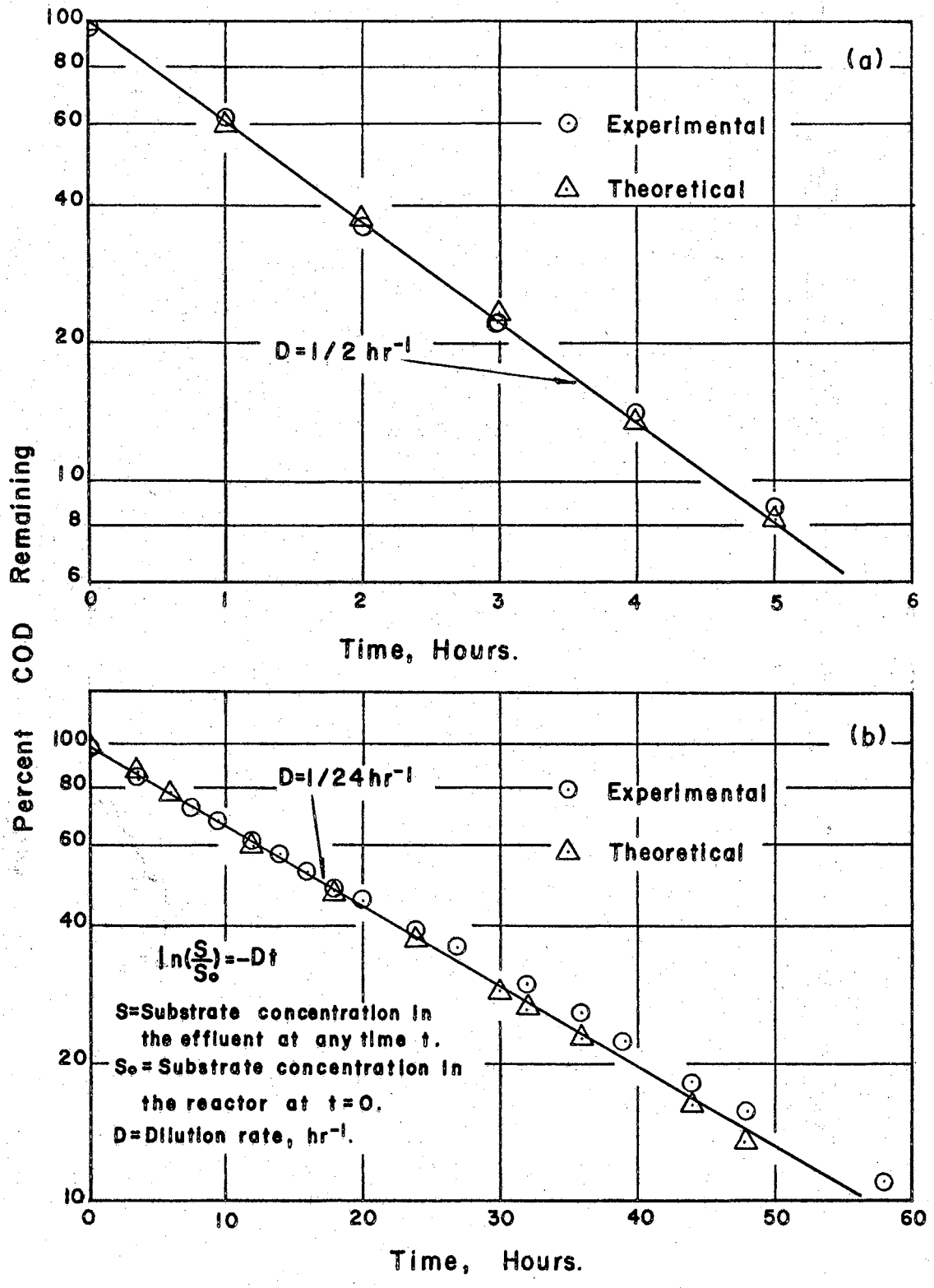

Figure 2 - Comparison of experimental and theoretical dilute-out curves at a dilution rate of 1/2 hr^{-1} and 1/24 hr^{-1}.

The reactors used in the long-term batch experiments were 4-liter Ehrlenmeyer flasks with two gas dispersion tubes in each to provide adequate mixing and aeration.

In all experiments, compressed air was saturated with water by bubbling it through distilled water prior to its entry to the flask. Airflow rate was maintained at 2000 ml/min/l which provided more than 6 mg/l of DO (dissolved oxygen) concentration in the reactor. At times, when filamentous organisms and side growth occurred and prevented complete mixing in the continous flow reactor, the aeration rate was increased to 3000 ml/min/l.

b. Heterogeneous Populations

The organisms employed in all experiments were developed from fresh sewage obtained from the primary clarifier effluent of the sewage treatment plant in Stillwater, Oklahoma.

In the short-term batch experiments, the microbial seeds were developed by the following acclimation process: fifty ml of the standard synthetic medium containing 1000 mg/l of the substrate to which the sludge would be acclimated were inoculated with approximately 5 ml of fresh sewage. The mixed liquor was placed in a 250 ml Ehrlenmeyer flask and aerated at room temperature on a reciprocal shaker at 100 strokes/min. After approximately 36 hours, 10 ml of the mixed liquor were transferred to 500 ml of fresh synthetic medium which had the same composition as that described above. Then this mixed liquor was distributed into a series of 250 ml Ehrlenmeyer flasks, and again aerated on the shaking apparatus. After approximately 18 hours, the carbon source was exhausted and the heterogeneous microbial populations were harvested to seed the experimental reactors.

The development of the heterogeneous populations employed in the continuous flow experiments was the same as for short-term batch experiments except for the carbon source. In this case the combined substrate system under study was used instead of a single substrate.

For long-term batch experiments, the heterogeneous microbial seed was developed by the following procedures. One thousand mg/l of glucose growth medium were used to grow the organisms in a 1.5-liter batch reactor. After approximately each 24 hours of aeration, one-third of the mixed liquor was wasted and the remainder was settled for one hour. Following the settling period, another one-third of the liquor was wasted, and then the mixed liquor was made up to 1.5 liters with growth medium. After attainment of solids balance, the sludge was harvested by centrifuging the mixed liquor and washing twice in the growth medium devoid of glucose and $(NH_4)_2SO_4$. Then the washed cells were blended for ten seconds in a Waring blender and suspended in the washing medium. This suspension was used to seed the reactors.

C. Experimental Protocol

a. Short-term Batch Experiments

In the three-component studies the experimental system consisted of three single-substrate reactors and one combined substrate reactor containing three carbon sources. Similarly, the four-component systems consisted of four control units, one for each substrate, and one unit for the mixed components. Five hundred mg/l of the specific carbon source were added to the appropriate control reactor, whereas the combined reactor received 500 mg/l of each substrate. At the beginning of the experiment, 100 ml of the acclimated suspension cells were added to

each reactor and made up to 1.5 liters with growth medium. The batch reactors were aerated vigorously by compressed air. Twenty-five ml samples were taken periodically from each reactor and centrifuged for 10 to 15 minutes at 12,000 rpm in a Sorvall SS-1 centrifuge. At the same time, samples were removed for the assessment of biolgical growth by measuring the optical density. After centrifugation, the supernatant was passed through a Millipore filter (HA, 0.45 μ pore size) into a collection tube. Either 5, 10, or 20 ml of the collected filtrates were used for COD determination, and the remaining volume was frozen for later analysis for specific substrates.

b. Continuous Flow Experiments

Before starting continuous flow operation, 2.3 liters of the synthetic waste containing the desired mixed substrate, 1500 or 2000 mg/l COD, were inoculated with 200 ml of the microbial population which had been previously acclimated to the growth medium.

At zero hour, the aeration was begun and the pump was set at the lowest flow rate ($D = 1/24$ or $1/12\ hr^{-1}$); the substrate concentration in the feed was maintained constant at all times. During transient states, samples were collected periodically to determine the system responses resulting from changes in dilution rate. The number of samples collected in the transient state was governed by the severity of the system disruption caused by the hydraulic shock loading. As the system came into a steady-state, a sufficient number of less closely-spaced samples was taken to assess the steady-state behavior of the system.

For each sampling period, a 100-ml beaker, immersed in ice, was employed to collect the reactor effluent. Two 25-ml samples of

mixed liquor were withdrawn from the beaker, centrifuged immediately, and filtered as described earlier. After centrifugation, the supernatant was carefully removed from the tubes, 10 or 20 ml were used for COD determination, and a 20-ml portion of the remaining supernatant was frozen for later analysis for specific carbon sources and metabolic intermediates. The remainder of one of the samples was used to measure the biological solids concentration.

The degree of complete mixing with respect to suspended solids was assessed at frequent intervals by comparing the optical density of the reactor mixed liquor and the reactor effluent. Identical or nearly identical OD readings were taken as evidence of complete mixing. When the biological solids concentration (optical density) remained fairly steady, the system was adjudged to be in the steady state.

Upon the attainment of steady-state, the dilution rate was increased to a higher dilution rate (i.e., D = 1/24, 1/12, 1/8, 1/6, 1/4, 1/3, 1/2.5, then 1/2 hr^{-1}). The experiment was terminated when the system exhibited nearly complete dilute-out of cells and substrate (the highest dilution rate).

c. Long-term Batch Experiments

Before starting the experiments, the microbial population developed earlier was divided into four 100-ml portions. One portion was used to seed a reactor (3 liters total reaction liquor) which contained all of the nutrients in excess but a limiting amount (3000 mg/l) of the carbon source (glucose). This system was hereinafter referred to as the "growing system." The other portions were employed to seed three other reactors which contained all of the nutrients of the growth medium

except nitrogen. These units were referred to as the "nonproliferating systems."

Initial samples were withdrawn from each system after thorough mixing, and aeration was then begun. Beginning on the second day of the experiment, the volume of the mixed liquor was measured before each sampling, and the evaporation loss was made up with distilled water. During this operation, care was taken not to lose any mixed liquor.

The sampling procedures for the long-term batch experiments were the same as for short-term batch experiments, except that analysis for biological solids concentration and cell components (protein and carbohydrate) were made. Two 20-ml samples were taken from each reactor; the biological solids were collected from one of the samples and resuspended in a 25-ml vial with 10 ml of distilled water. The suspension was frozen immediately and stored at -15°C for later analysis for cell carbohydrate and protein. The procedures employed were those recommended by Gaudy (45).

D. Analytical Techniques

The analytical techniques were the same for both batch and continuous flow experiments except where noted.

a. Biological Solids Determination

In the continuous flow and the long-term batch experiments, the biological solids concentration of the liquor was determined by the membrane filter technique (0.45 μ pore size, Millipore Filter Corp., Bedford, Mass.) according to Standard Methods (46). The optical density (at 540 mμ) of the cell suspension was used to determine the biological growth in the short-term batch experiment. All of the

optical density determinations used in this study were performed with a Bausch & Lomb Spectronic 20 spectrophotometer with 1/2-inch matched test tubes.

b. Chemical Analyses

The total COD of the membrane filtrate was measured in accordance with the procedure given in "Standard Methods" (46). Mercuric sulfate and silver sulfate were used for all COD determinations. Occasionally the substrate concentration of the continuous flow feed was also determined by COD test as a check on the stock feed solution.

For glucose concentration in the filtrate, the enzymatic Glucostat test was employed. The test was performed in accordance with Method 1-a of the manufacturer's specification (47).

Galactose concentrations in the filtrates were determined colorimetrically by the Galactostat method (48).

Concentrations of pentoses (ribose and xylose) in the membrane filtrates were measured by the orcinol test as described in "Experimental Biochemistry" (49).

The resorcinol method of Roe for ketohexoses (50) was employed as an analysis for fructose and sucrose.

Sorbitol, glycerol, and periodate-reactive metabolic intermediates were determined by the periodate oxidation method of Neish (51). An oxidation time of ten minutes was adopted in this study to allow a more complete oxidation (52).

The carbohydrate content of the filtrate or of the solids suspension was measured by the anthrone method, as outlined by Gaudy (45). The solids suspension was homogenized by sonic oscillation (Sonifier, Branson Sonic Power, Danbury, Conn.), and then an aliquot of the

homogenized suspension was analyzed for cellular carbohydrate, using glucose as the standard.

The protein content of the homogenized solids suspension was estimated by the Biuret test, as recommended by Gaudy (45). This procedure was adopted for sludge protein concentration within the range of 1 to 10 mg. For sludge samples containing concentrations below one mg, the Folin-Ciocalteau test (49) was employed. Crystalline, grade A bovine plasma albumin was used as the standard for these two tests.

The concentration of pyruvic acid (keto acid) in the membrane filtrate was determined by the method of Friedeman and Haugen (53) as modified by Hamilton, et al. (54). The optical density was measured at 540 mμ. Pyruvic acid was used as the standard.

c. Gas-liquid Chromatography

Volatile acids were identified and measured by gas-liquid chromatography. A model 810 "Research Chromatograph" (F & M Scientific Division, Hewlett Packard Company, Avondale, Pa.) equipped with hydrogen flame detectors and a Minneapolis-Honeywell recorder was used for this analysis. The column used in the experiments was a 74-inch precoiled glass tube of 3/16-inch inside diameter packed with a porous polymer (Polypak-2) of 80-120 mesh size (Hewlett Packard Company, Avondale, Pa.), which was thermally stable up to 300°C in an oxygen-free atmosphere.

The following analytical conditions were employed for the determination of acetic acid, propionic acid, and butyric acid:

Airflow rate @ 20 psi	295 ml/min
Helium flow rate @ 60 psi	50 ml/min
Hydrogen flow rate @ 10 psi	28 ml/min
Flame detector temperature	285°C

Oven temperature	190°C
Injection port temperature	275°C
Volume of sample injected	5 µl

It is known that the sequential elution of the volatile acids is dependent on increasing numbers of carbon atoms. The retention times for standards of the individual acids were approximately 3 minutes for acetic acid, 5.5 minutes for propionic acid, and 10 minutes for butyric acid; standards were run frequently. The peak area method was used to determine the concentration of the identified acids.

d. Dissolved Oxygen Determination

Dissolved oxygen concentrations in the experimental reactors were occasionally measured by an electrometric technique, using a galvanic cell oxygen analyzer (Precision Scientific Company, Chicago, Ill.). Checks on the dissolved oxygen were made to ensure that the system was operating under strictly aerobic conditions.

CHAPTER IV

RESULTS

All data obtained during the batch experiments in these investigations were subjected to kinetic analyses, i.e., computations for logarithmic growth rate constant, COD and substrate removal rate constants and sludge yield were performed. The constancy of sludge yield throughout the substrate removal phase was examined by plotting COD removal versus biological solids concentration. The results of these calculations in some cases have direct bearing on the manifestations of metabolic control mechanism, and in some cases they do not aid in analyzing the mode of substrate removal in multicomponent media. Because of the many graphs and tables which would necessarily need to be shown if these data were included in this chapter, and because it seemed more desirable to present the actual experimental results here, the kinetic calculations, etc., are presented in the appendix.

As an aid to comparative analysis of the results, a more direct and conventient approach was sought. The method of comparative analysis selected was as follows: A COD concentration of 200 mg/l and a biological solids concentration equivalent to an optical density of 0.3 (approximately 175 mg/l) were chosen as "reference points." The selection of these values facilitated comparison of the rates of bacterial growth and COD removal in the control units with those in the combined

system. In general, these two "reference points" occurred in the log phase of growth. The expression "reference time" (RT) is used in this chapter to represent the time required to reach the "reference point." It is felt that this approach had three major advantages as an aid in analyzing and interpreting the results: (1) since in all systems the same cell and substrate concentrations were used, the "reference time" permits direct comparison of the actual results; (2) the use of the parameter "reference time" provides the reader with a convenient and rapid means of scrutinizing the author's interpretation and analysis of the results; (3) "reference time" provides a better overall comparative assessment for possible prediction of the order of substrate removal in multicomponent media from behavior in the control systems because it includes the effect of a lag or acclimation period as well as specific growth rate.

A. Studies on Substrate Removal in a Mixture of Glucose, Galactose, and Fructose by a Heterogeneous Microbial Population

I. Batch Experiments

a. Glucose-acclimated Cells

The growth and substrate utilization responses of a glucose-acclimated sludge to glucose, fructose, and galactose as single carbon sources are given in Figure 3. The observation that there was no lag in either fructose or galactose removal suggests that the cells possessed constitutive enzymes for both sugars. A comparison of the total COD and substrate COD values reveals that the relative amounts of exogenous metabolic products accumulated in these media during metabolism of the sugars were of the following order: glucose > galactose >

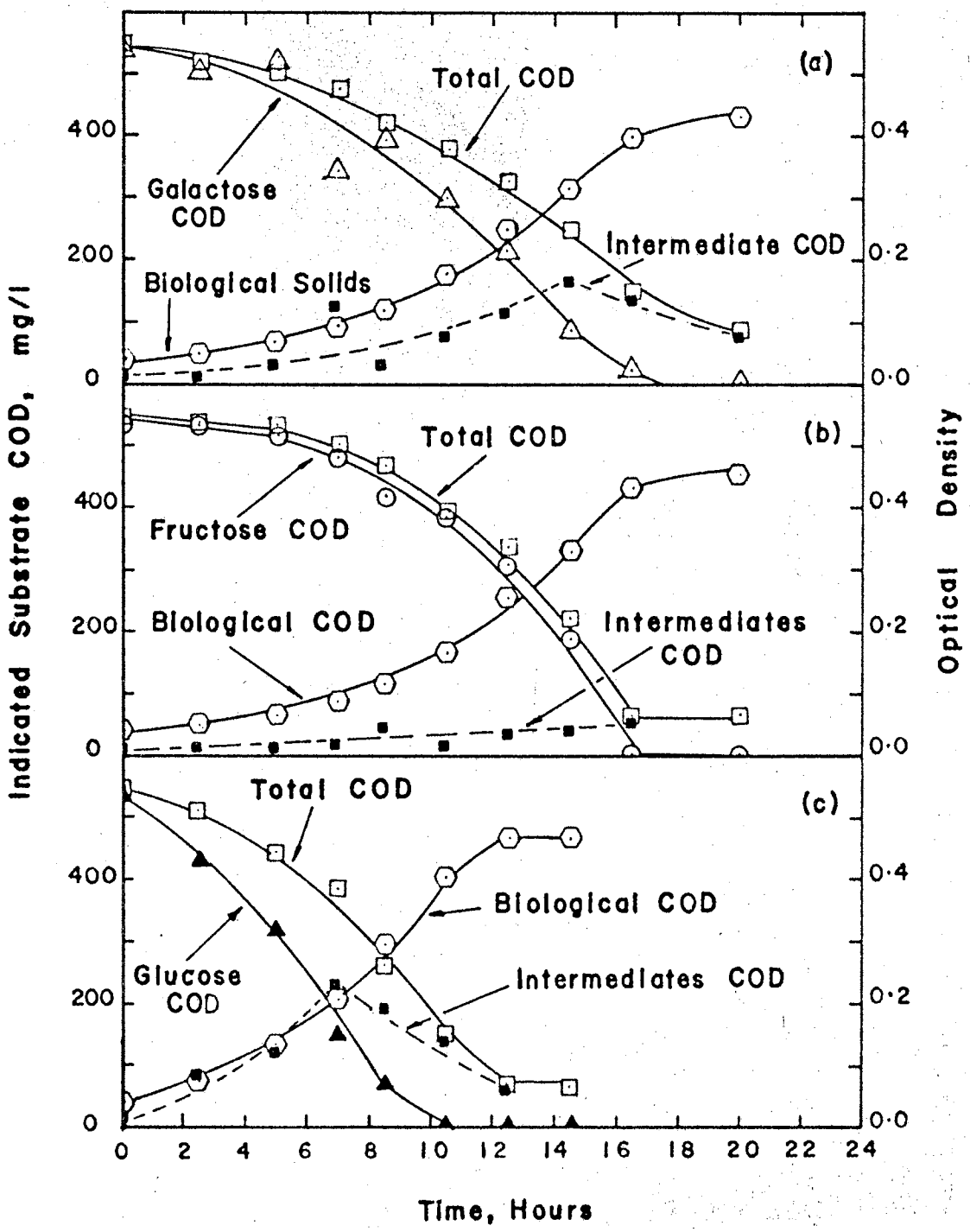

Figure 3 - System performance in the control units of (a) galactose, (b) fructose, and (c) glucose; young cells acclimated to glucose.

fructose. It can be seen that the required times for growth to O. D. = 0.3 (reference time) were 9 hours for glucose, 14.1 hours for fructose, and 14.2 hours for galactose; i.e., glucose supported faster growth than did fructose or galactose.

Figure 4 shows the growth curve and COD removal curves in the combined unit containing glucose, galactose, and fructose. Comparing the curves of bacterial growth and glucose removal in this unit with those in the glucose control, it can be seen that during the first eight hours the cells grew primarily on glucose, and the presence of galactose and fructose exerted no effect on glucose metabolism. Galactose removal did not start until 72 per cent of the glucose had been consumed, whereas fructose utilization did not start until all of the glucose was exhausted. This result indicates that the removal of galactose and fructose were hampered or blocked, due to glucose metabolism. This interference might be due to either the mechanism of catabolite inhibition or a combination of catabolite inhibition and repression. Since the times required to remove the glucose to a concentration of 200 mg/l ("reference time") were 6.8 hours in the glucose control and 6.5 hours in the combined unit, the glucose removal rates in these two units were much the same. Although fructose removal was initiated 1.5 hours after galactose, no interaction between these two sugars was apparent. The 1.5-hour delay of fructose removal might have been due to the effect of remaining glucose intermediates rather than to galactose.

The dashed curves in Figures 3 and 4 show the concentrations of exogenous metabolic intermediates in the reactor. The intermediate COD was calculated as the difference between total COD and the sum of the COD's of the sugars. The maximum production of intermediates

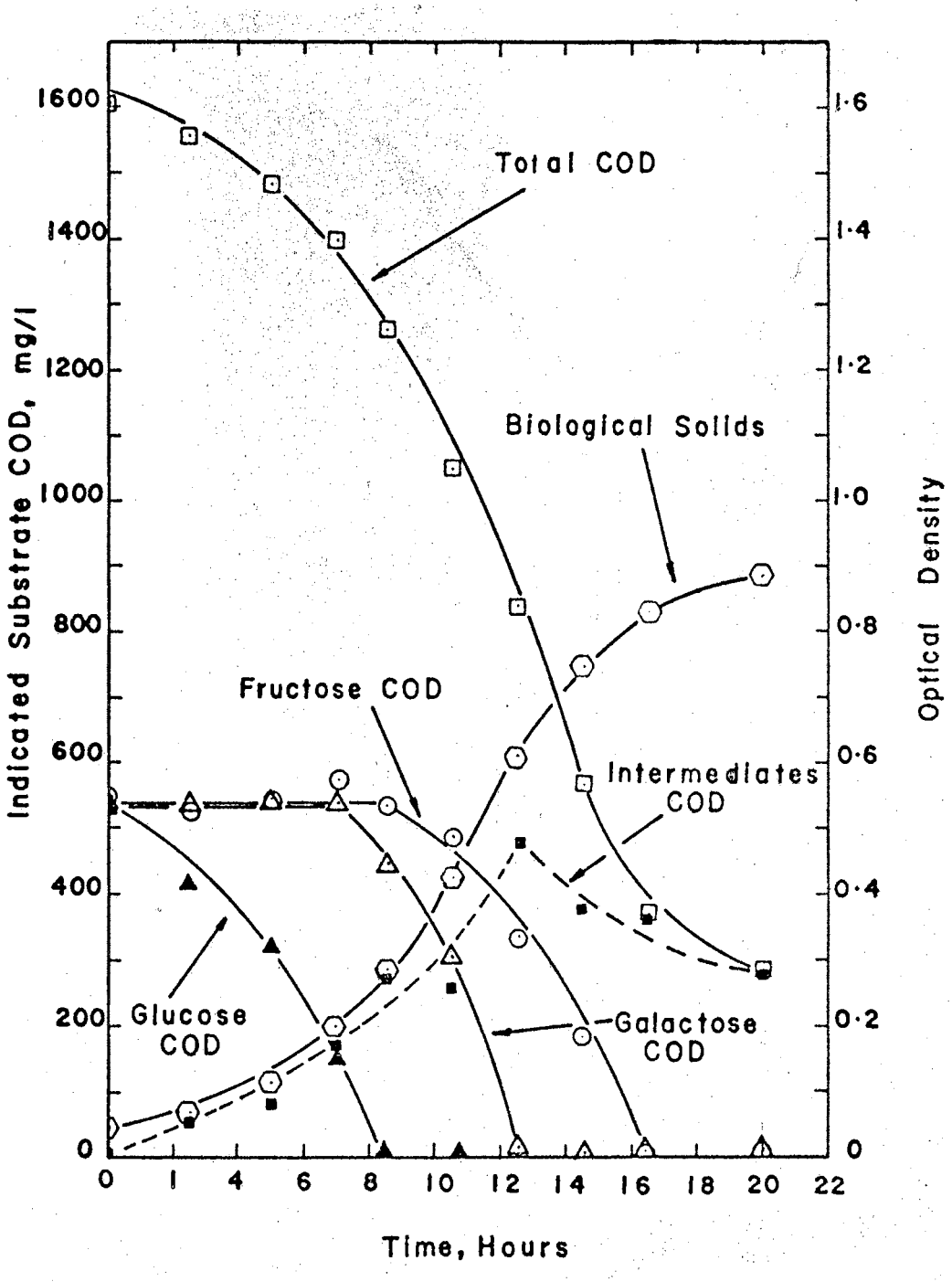

Figure 4 - System performance in the combined unit of glucose, galactose, and fructose; young cells acclimated to glucose.

attributable to glucose (270 mg/l) took place at the time of glucose exhaustion (8.5 hours) in the combined system, whereas 230 mg/l had accumulated at 7.0 hours in the control. The somewhat different patterns of intermediates accumulation in Figures 3 and 4 suggest that the presence of the other two sugars, especially galactose in this case, might have altered the character of intermediates accumulation, though the organisms apparently grew only on glucose during this period.

b. Galactose-acclimated Cells

The patterns of substrate removal and biological growth with each sugar as sole carbon source are presented in Figure 5. Plots of optical density versus time on semilogarithmic paper revealed that essentially no lag period was required for the galactose-grown cells to metabolize glucose and fructose. Thus the organisms contained the enzymes required for glucose and fructose metabolism. It is seen that glucose (RT = 6.0 hrs) was removed at a faster rate than were fructose (RT = 8.0 hrs) and galactose (RT = 8.6 hrs), even though the cells had been acclimated to galactose. It can also be seen that more metabolic intermediates accumulated from glucose and fructose catabolism than from galactose. All of these observations suggest that the relative potential of these three sugars for substrate interference was of the following order: glucose > fructose > galactose.

Figure 6 shows the growth and substrate utilization responses in the combined glucose, fructose and galactose system. A comparison of the glucose removal rates in the combined (RT = 6.2 hrs) and in the control (RT = 6.0 hrs) reactors indicates that glucose removal was possibly very slightly retarded in the presence of the other two sugars. The 7-hour blockage of galactose removal indicates that glucose

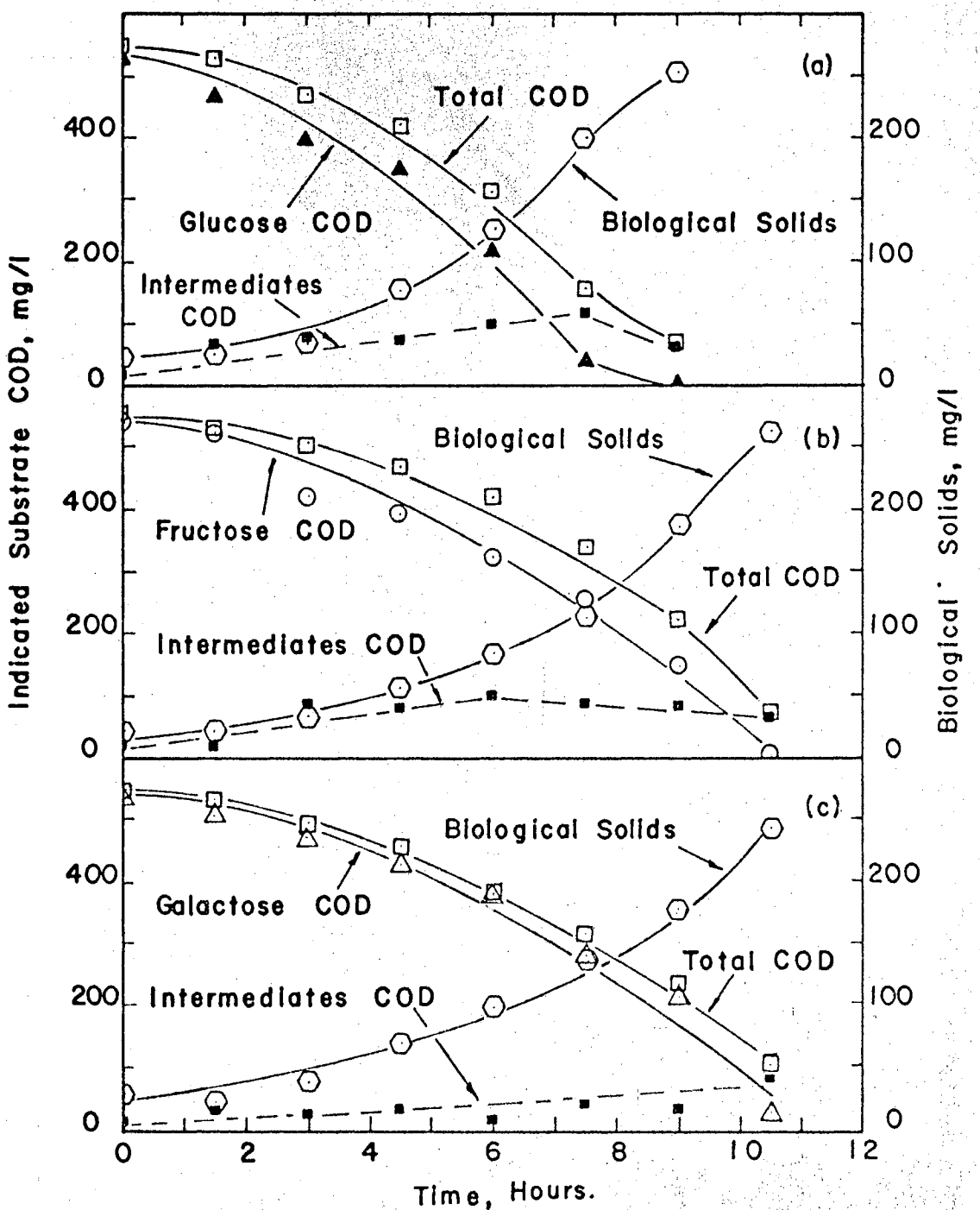

Figure 5 - System performance in the control units of (a) glucose, (b) fructose, and (c) galactose; young cells acclimated to galactose.

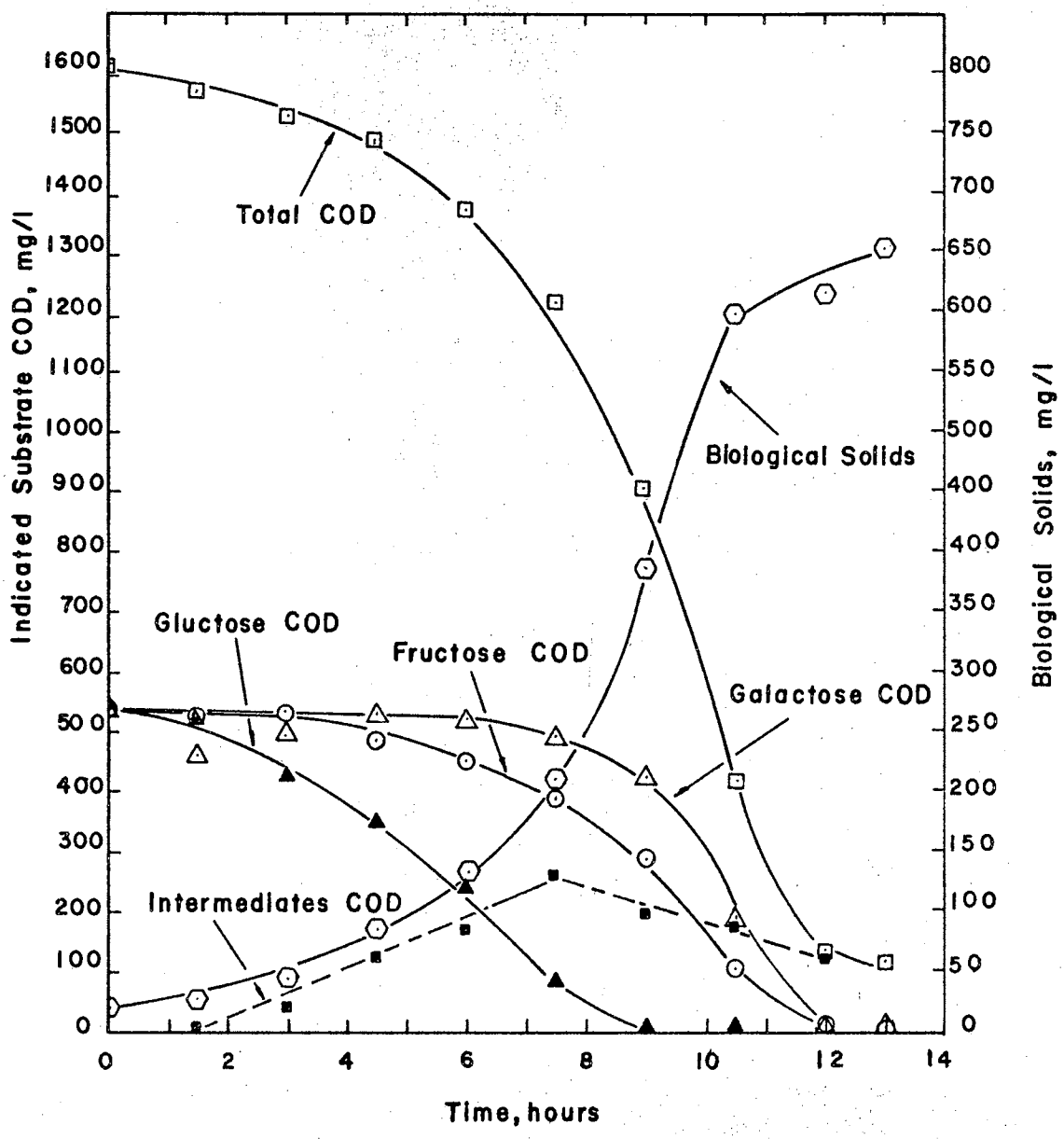

Figure 6 - System performance in the combined unit of glucose, galactose, and fructose; young cells acclimated to galactose.

severely inhibited the galactose-utilizing enzymes. The inhibition was not released until about 75 per cent of the glucose had been consumed; thus glucose appeared to cause 100 per cent inhibition of galactose enzymes when glucose concentration was higher than 25 per cent of the galactose concentration. The 3-hour pause in fructose removal in the combined system indicates that fructose-utilizing enzymes were subject to rather severe glucose inhibition when glucose concentration was higher than 81 per cent of the fructose. Again it appears that the amount of intermediates accumulating from glucose metabolism in the control system and in the mixed sugar system were not similar. The fact that galactose removal was initiated at the time the highest amount of intermediates was present in the reactor suggests that the glucose itself might also play an important role in galactose inhibition, i.e., inhibition may not be due only to the catabolites produced from glucose metabolism. By comparison of galactose removal rates in the control (RT = 8.6 hrs) and the combined units (RT = 10.5 hrs), it is seen that the galactose removal rate was enhanced greatly after release from the 6-hour glucose inhibition. Enhancement of galactose removal was also evident in Figure 4. However, from these observations it cannot be concluded that the presence of fructose enhanced the galactose removal rate, since the more rapid removal of galactose was most probably due to the higher concentration of organisms which were produced during the period of glucose metabolism in the combined reactor. The substrate removal curves in Figure 6 show that the order of priority in utilization was glucose > fructose > galactose. Thus the order of removal was in accordance with the relative potential for interference based upon growth rate and accumulation of intermediates in the control reactors.

c. Fructose-acclimated Cells

Figure 7 shows the substrate removal and growth responses of a fructose-acclimated microbial population when glucose, fructose, and galactose were used as sole sources of carbon. From semilogarithmic plots of optical density and substrate removal it was discerned that there was little or no lag in either glucose or galactose metabolism, suggesting that the cells did not require acclimation to these two sugars. The "reference times" for biological growth were 6.4 hrs for glucose, 7.2 hrs for fructose, and 10.5 hrs for galactose. From Figure 7 it can be seen that the accumulation of intermediates from the three substrates followed the order: glucose > fructose > galactose. Therefore, it is logical to propose that the relative potential for substrate interference is: glucose > fructose > galactose.

Figure 8 shows the utilization of substrates when glucose, galactose, and fructose were used as combined carbon source. A comparison of glucose removal in the control (RT = 6.4 hrs) and the combined system (RT = 6.5 hrs) indicates that the presence of the other two sugars did not significantly interfere with the consumption of glucose. However, the first phase of growth in the combined system (RT = 5.8 hrs) was faster than that in the glucose control (RT = 6.4 hrs). There was no fructose removal until 38 per cent of the glucose had been consumed. This indicates that fructose enzymes were 100 per cent inhibited when glucose concentration was greater than two-thirds that of fructose. The linear fructose removal following the 5-hour blockage suggests that the fructose was being removed by pre-formed enzymes and that no additional synthesis of a fructose-degrading enzyme system occurred. The linear rate was not a slow one, suggesting that there was an

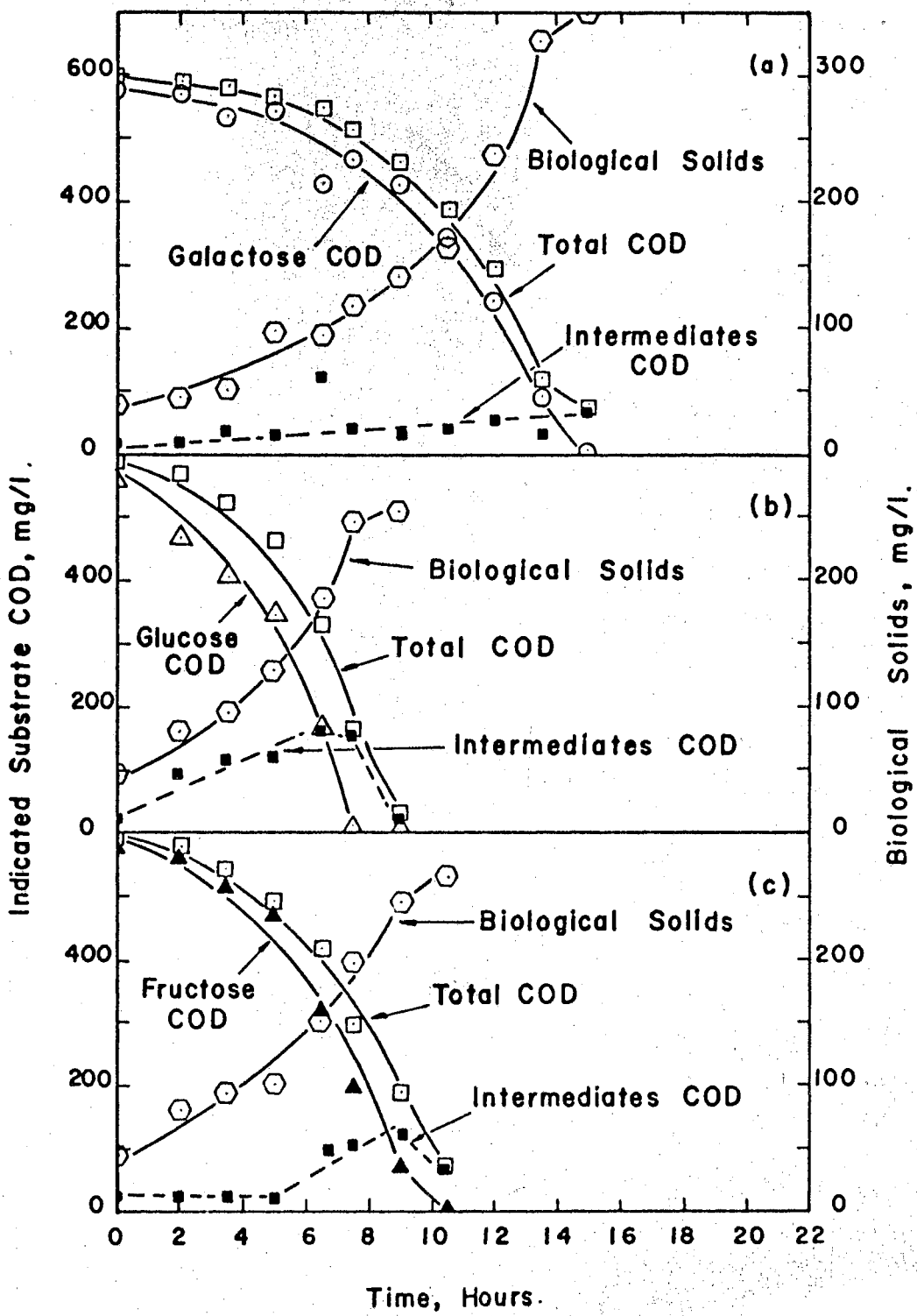

Figure 7 - System performance in the control units of (a) galactose, (b) glucose, and (c) fructose; young cells acclimated to fructose.

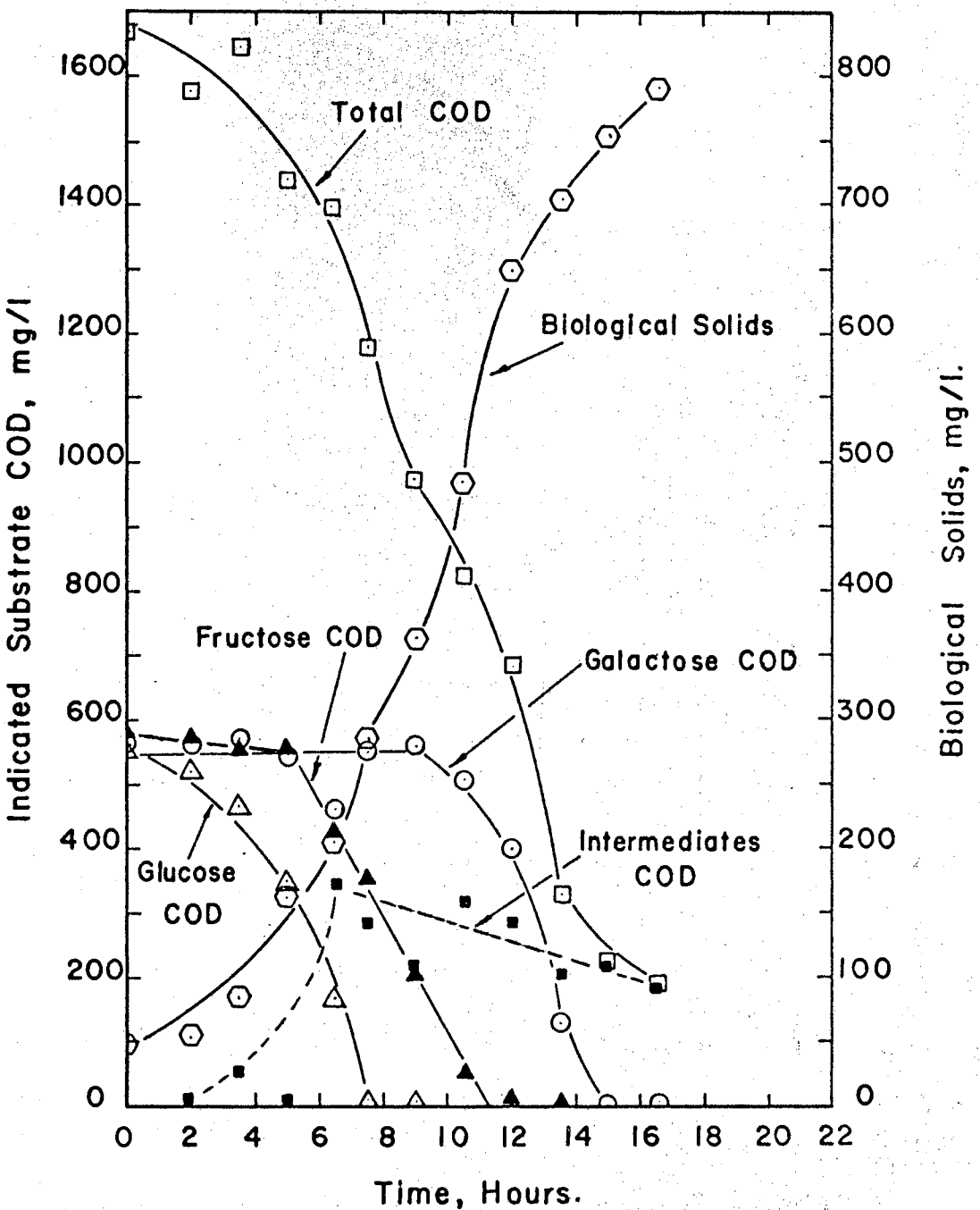

Figure 8 - System performance in the combined unit of glucose, galactose, and fructose; young cells acclimated to fructose.

increase in the amount of enzymes required for fructose utilization during the course of glucose removal. If this is true, the 5-hour pause in fructose removal was attributable to complete inhibition of enzymes as they were formed, not to repression of new enzyme formation. In the control, if the fructose-COD data are fitted to a straight line, using the samples taken between 5 and 9 hours, it can be seen that the fructose removal rate was 100 mg/l/hr. This indicates that the linear removal of fructose in the combined system (K_f = 88 mg/l/hr) was subject to a small degree of inhibition by glucose.

The fact that galactose removal did not start immediately at the time glucose was exhausted provides some indication that galactose removal was subject to inhibition by fructose or accumulated intermediates. The results of previous experiments (Figures 4 and 6) suggest that it is unlikely that the continued inhibition of galactose was due to the remaining fructose. It is seen in Figure 8 that the two growth phases (μ_1 = 0.350 hr^{-1}, μ_2 = 0.172 hrs^{-1}) corresponded to diphasic total COD removal (K_1 = 0.324 hr^{-1}, K_2 = 0.143 hr^{-1}). The second phase in the total COD curve was due largely to removal of galactose and metabolic intermediates. The sequence of removal is consistent with the relative potential sequence, i.e., glucose > fructose > galactose, observed in the controls.

It is interesting to note that glucose retarded galactose utilization to about the same extent whether the cells were acclimated to glucose (Figure 4) or galactose (Figure 6); i.e., galactose removal was initiated at about 7 hours in both systems. Since this result in Figure 6 is ascribable to inhibition of the activity of existing enzymes, the result in Figure 4 may indicate that the presence of glucose did

not prevent synthesis of galactose utilizing enzymes but did inhibit their function.

II. Continuous flow Experiments

After obtaining information on the batch growth of natural populations in glucose, galactose, and fructose, the mixed substrate system was investigated under continuous culture conditions. The organisms used to seed the continuous flow reactor were grown on initial sewage seed on a mixture of these three sugars. Beginning at a low dilution rate, at least six mean residence times were allowed to elapse before the dilution rate was increased. The results for this continuous operation are presented chronologically, from low dilution rates to high dilution rates, in Figure 9.

It is seen that upon addition of the cells, the substrates were rapidly removed and the biological solids concentration attained a fairly steady level. Only trace quantities of glucose, fructose, and galactose were detected, and COD removal efficiency was rather good. When the dilution rate was changed from 1/12 to 1/8 hr^{-1}, there was no transient leakage of substrates, and the effluent characteristics were essentially the same for both dilution rates. The biological solids concentrations were abnormally high at 159 hours (1240 mg/l) and at 168 hours (1160 mg/l). During this period of operation the unit could not be considered to be completely mixed, as evidenced by higher biological solids concentration in the reactor than in the effluent. At this time there had occurred a decided change in species predominance, as evidenced by the development of cells which existed as large, loosely-bound flocs, and the mixed liquor exhibited a light pink color. After 60 hours of operation at this flow rate (180 hours on the time scale),

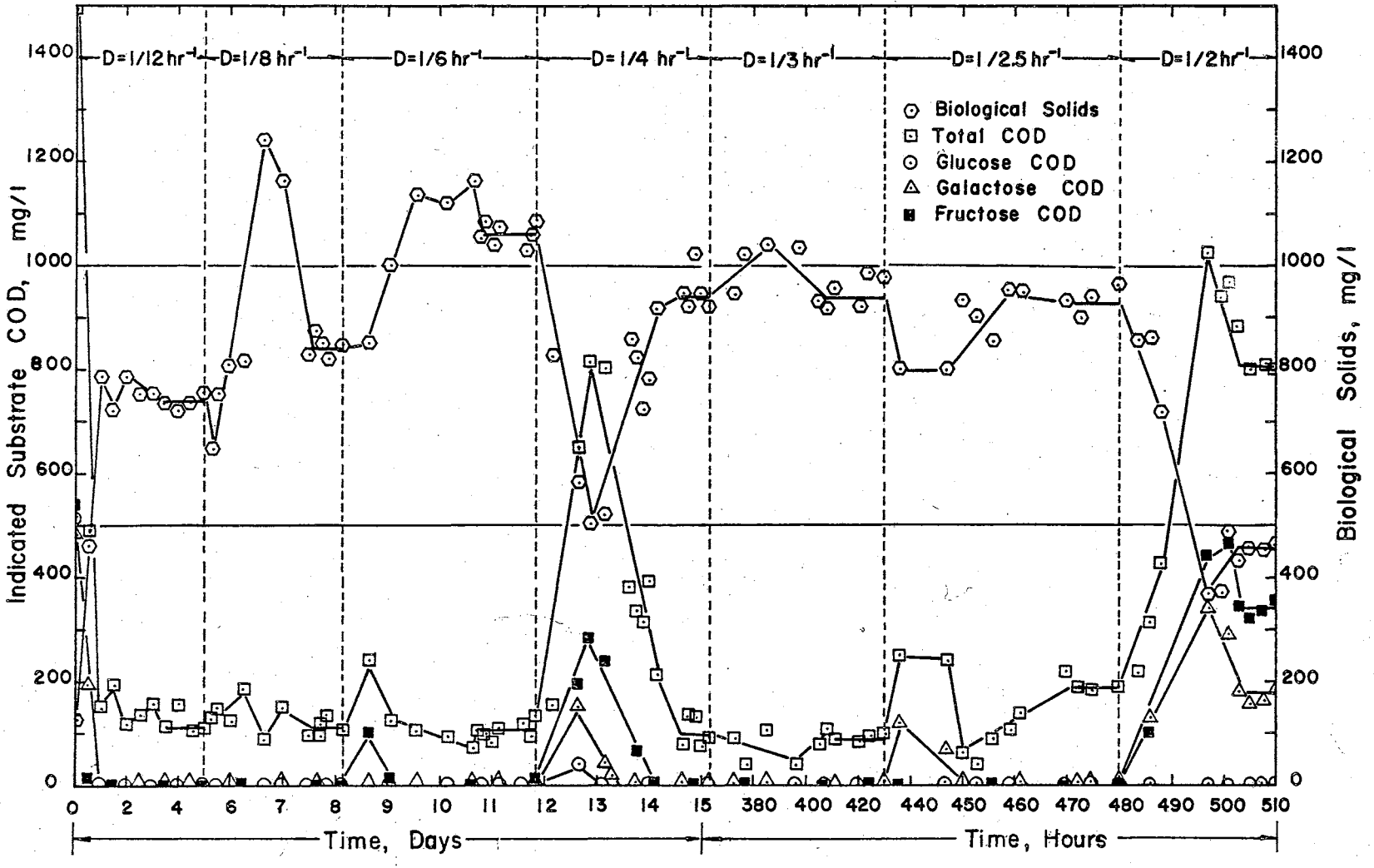

Figure 9 - System performance in the continuous flow activated sludge unit growing on glucose, galactose, and fructose at various dilution rates.

the biological solids level had dropped sharply, and most of the loose flocs had disappeared from the reactor. When the new steady-state was attained and complete mixing was in evidence, the biological solids level remained fairly constant at approximately 840 mg/l. The mixed liquor retained a pink hue but of greatly decreased intensity.

When the dilution rate was changed to 1/6 hr^{-1}, there was a transient leakage of 100 mg/l fructose. It is interesting to note that a sharp increase in biological solids concentration occurred and was accompanied by a change in predominance. By the time fructose concentration returned to the previous steady-state value (219 hours), the mixed liquor exhibited a milky white color. When the unit approached a new steady-state, the biological solids concentration was 1060 mg/l, indicating a cell yield of approximately 71 per cent, and substrate removal was again as efficient as it had been at the previous dilution rate (93 per cent).

A very severe disruption of substrate removal efficiency was caused by the change of dilution rate from 1/6 hr^{-1} to 1/4 hr^{-1}. Within 24 hours after shifting the dilution rate, the biological solids concentration dropped from 1060 mg/l to 510 mg/l, while the COD increased from 110 mg/l to 810 mg/l. The maximum substrate leakages during this period were: fructose, 280 mg/l, galactose, 150 mg/l, and glucose, 45 mg/l. Also it appeared that the shock caused a change of the predominating species in the reactor. During cell washout the color of the reaction liquor changed from milky white to light brownish-gray. When the system reached a new steady-state, the biological solids concentration rose, the effluent COD returned to approximately 100 mg/l, and the individual substrates were not detected in the filtrate.

No significant changes of COD and solids levels occurred when the dilution rate was increased to 1/3 hr^{-1}.

During the transient response when the dilution rate was changed from 1/3 to 1/2.5 hr^{-1}, the solids concentration decreased from 940 mg/l to 800 mg/l, while the total COD in the effluent rose from 90 mg/l to 250 mg/l. Half of this COD was attributable to galactose. The system then recovered, and by the 40th hour after applying the hydraulic shock loading (470 hours), a new steady-state condition was attained. The steady-state biological solids concentration returned to its previous steady-state level, but the effluent COD was higher (190 mg/l; 85 per cent efficiency).

A severe disruption of substrate removal and a rapid washout of biological solids occurred when the dilution rate was changed from 1/2.5 to 1/2 hr^{-1}. After attainment of a new steady-state, glucose was absent from the medium, but significant residual steady-state concentrations of fructose (340 mg/l) and galactose (180 mg/l), as well as a considerable amount of metabolic intermediates, were present.

The steady-state levels of the various parameters at each dilution rate are shown in Figure 10. Analyses for metabolic intermediates are shown in the upper portion of the figure. The steady-state solids, total COD and substrate concentrations are the average of the steady-state values shown in Figure 9. Similarly, the concentration of metabolic intermediates is the average value of steady-state intermediates at each dilution rate. It appears that glucose is the preferred substrate, and in no case was glucose found in the effluent. At the dilution rate of 1/2 hr^{-1}, the efficiency of utilization galactose was reduced to 64 per cent, while only 36 per cent of the fructose in the

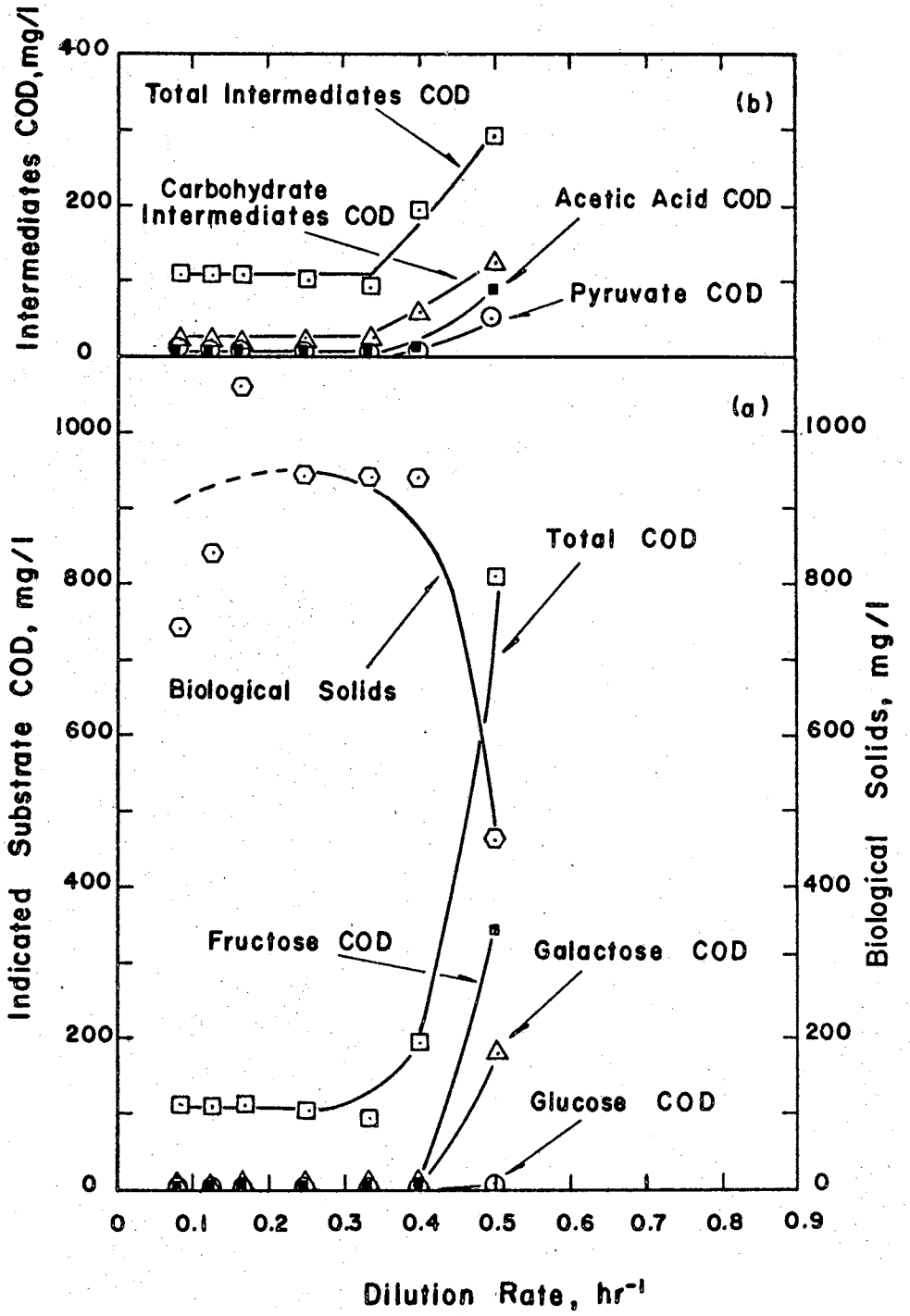

Figure 10 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, galactose, and fructose at various dilution rates; (b) intermediates accumulation.

feed was removed.

A substantial excretion of metabolic intermediates was observed at the dilution rate of 1/2 hr^{-1}. Nearly all of the intermediates (290 mg/l) could be accounted for as carbohydrate (120 mg/l), acetic acid (90 mg/l), and pyruvate (55 mg/l).

B. Studies on Substrate Removal in a Mixture of Glucose, Sorbitol, and Ribose by a Heterogeneous Microbial Population (Batch Studies Only)

a. Glucose-acclimated Cells

Figure 11 shows the substrate removal characteristics for glucose, sorbitol, and ribose by glucose-acclimated sludge in the individual control units. The long lag period for ribose utilization indicates that glucose-pregrown organisms did not possess constitutive enzymes for ribose metabolism at the start of the experiment and that these enzymes were not rapidly induced. The dashed curves (intermediate COD) suggest that more metabolic intermediates were excreted from glucose and sorbitol consumption than from ribose. The organisms grew on glucose (RT = 8.0 hrs) and sorbitol (RT = 14 hrs) more rapidly than on ribose (RT = 37 hrs). This was also borne out by the semilogarithmic plots. Therefore, based upon growth rate, intermediates accumulation and the need for acclimation to ribose, it is reasonable to propose that the relative potential for interference or order of removal in a mixture of the three substrates would be glucose > sorbitol > ribose.

Substrate utilization and growth in a medium containing glucose, sorbitol, and ribose are shown in Figure 12. It is seen that the organisms did not use sorbitol and ribose for growth when glucose was present in the medium. The first phase of growth in the combined reactor (RT =

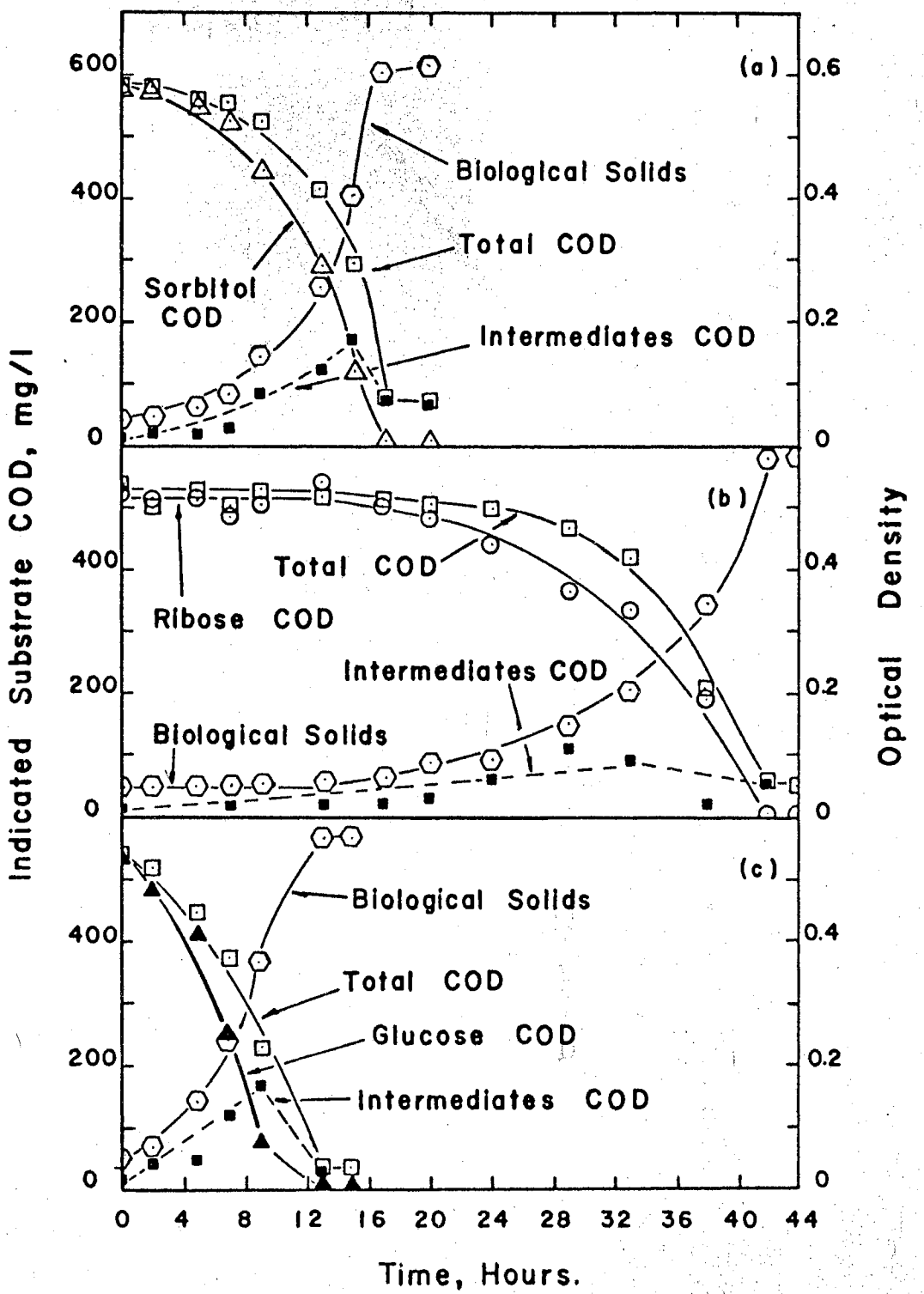

Figure 11 - System performance in the control units of (a) sorbitol, (b) ribose, and (c) glucose; young cells acclimated to glucose.

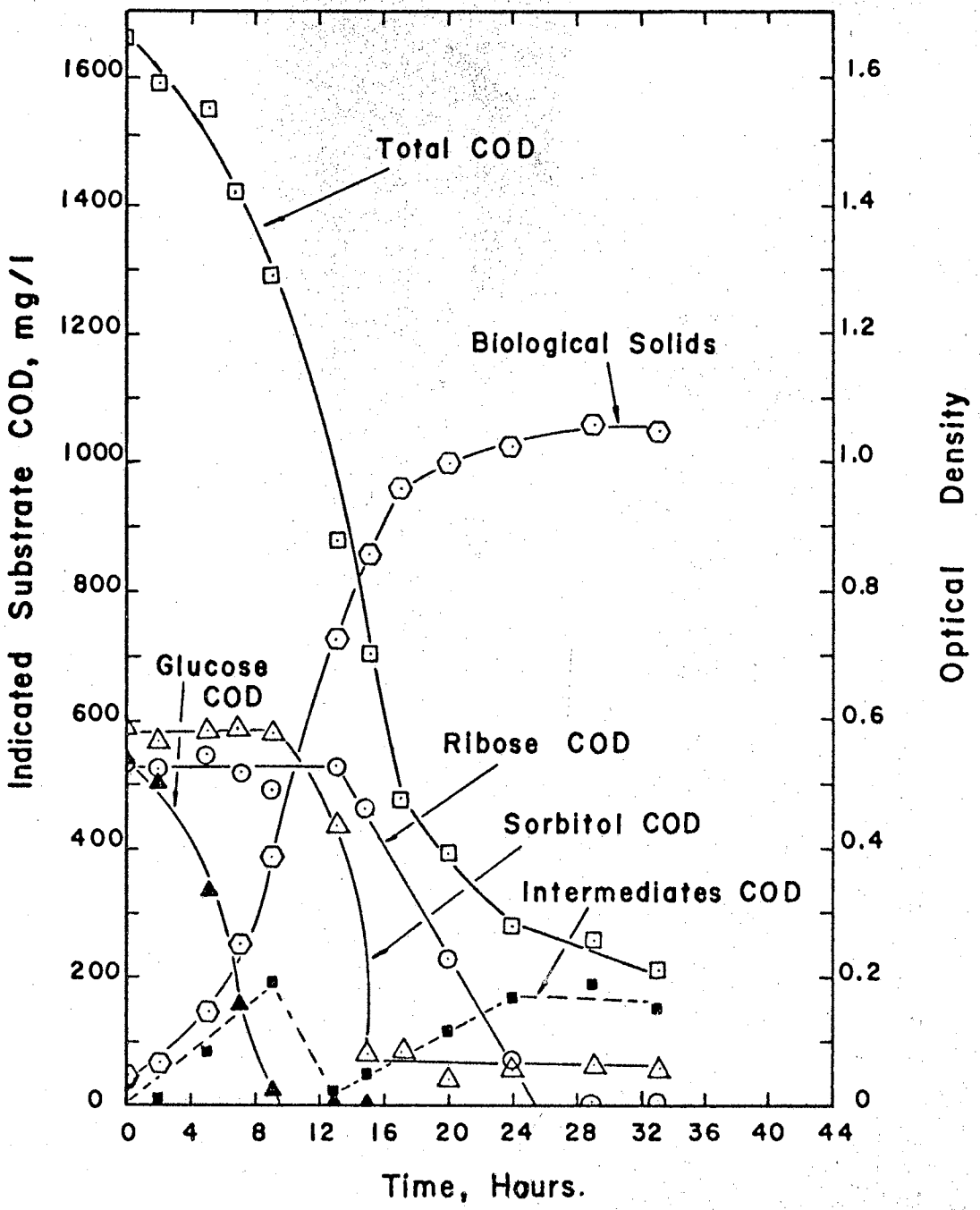

Figure 12 - System performance in the combined unit of glucose, sorbitol, and ribose; young cells acclimated to glucose.

7.6 hrs) was slightly more rapid than that in the glucose control (RT = 8.0 hrs). A comparison of the glucose removal rates in the combined (RT = 7.0 hrs) and in the control (RT = 7.6 hrs) systems indicates that glucose removal was only slightly (if at all) enhanced in the presence of the other two substrates. The removal of glucose was accompanied by a gradual accumulation of metabolic intermediates. It is also interesting to note that the time of glucose consumption corresponded to the time of maximum intermediates accumulation. The 9-hour blockage of sorbitol removal indicates that glucose severely suppressed the removal of sorbitol. A comparison of sorbitol removal in the control (RT = 14.5 hrs) and in the combined system after glucose was exhausted (RT = 6.0 hrs) suggests that a large percentage of enzymes required for sorbitol removal may have been synthesized during the period of glucose utilization. Therefore, the blockage of sorbitol removal was most likely due to severe inhibition of enzyme activity. The fact that the lag in ribose removal in the combined systems was equal to that in the control reactor (13 hours) illustrates that the synthesis of ribose enzymes was not affected (or induction time increased) by the presence of glucose or sorbitol. From this figure it is noted that sorbitol and ribose were removed concurrently and that ribose removal rate was not increased as soribtol concentration decreased. These data do not provide sufficient information to draw a definite conclusion concerning the interaction between these two carbon sources. Abput 60 mg/l to 70 mg/l of residual periodate-reactive material persisted in the medium. This material might have been produced during the metabolism of glucose or sorbitol. Since the linear removal of ribose did not enhance the buildup of the periodate-reactive intermediates, it seems that ribose did not contribute

to the periodate-reactive residual organic material.

In this study, the substrate removal curves (Figure 12) show that the priority order of consumption was glucose > sorbitol > ribose. The sequence is consistent with the relative potential sequence which was predicted on the basis of results for the control units.

b. Sorbitol-acclimated Cells

The curves for the control units for glucose, sorbitol, and ribose are presented in Figure 13. It can be seen that a 14-18-hour acclimation period was required for the utilization of ribose. Thus the sorbitol-acclimated cells did not possess a functioning enzyme system for ribose removal at the start of the experiment; the ribose enzymes were inducible, not constitutive, in these organisms. It is seen that growth on sorbitol (RT = 5.9 hrs) was slightly more rapid than on glucose (RT = 5.3 hrs), and much more rapid than on ribose (RT = 25.6 hrs). The relative amounts of intermediates accumulated in the reactors during metabolism of these substrates was of the following order: ribose > sorbitol > glucose.

The growth and substrate utilization responses in the combined glucose, sorbitol and ribose system are shown in Figure 14. A comparison of glucose removal rates in the combined (RT = 6.9 hrs) and the control (RT = 7.5 hrs) systems indicates that glucose removal was enhanced to some extent by the presence of the other substrates. The results indicate that there was a very severe disruption of sorbitol removal during the course of glucose removal. The lag in ribose in the combined reactor was significantly shorter than that in the control, indicating that the synthesis of required ribose enzymes was enhanced by the presence of the other two carbon sources and that during removal

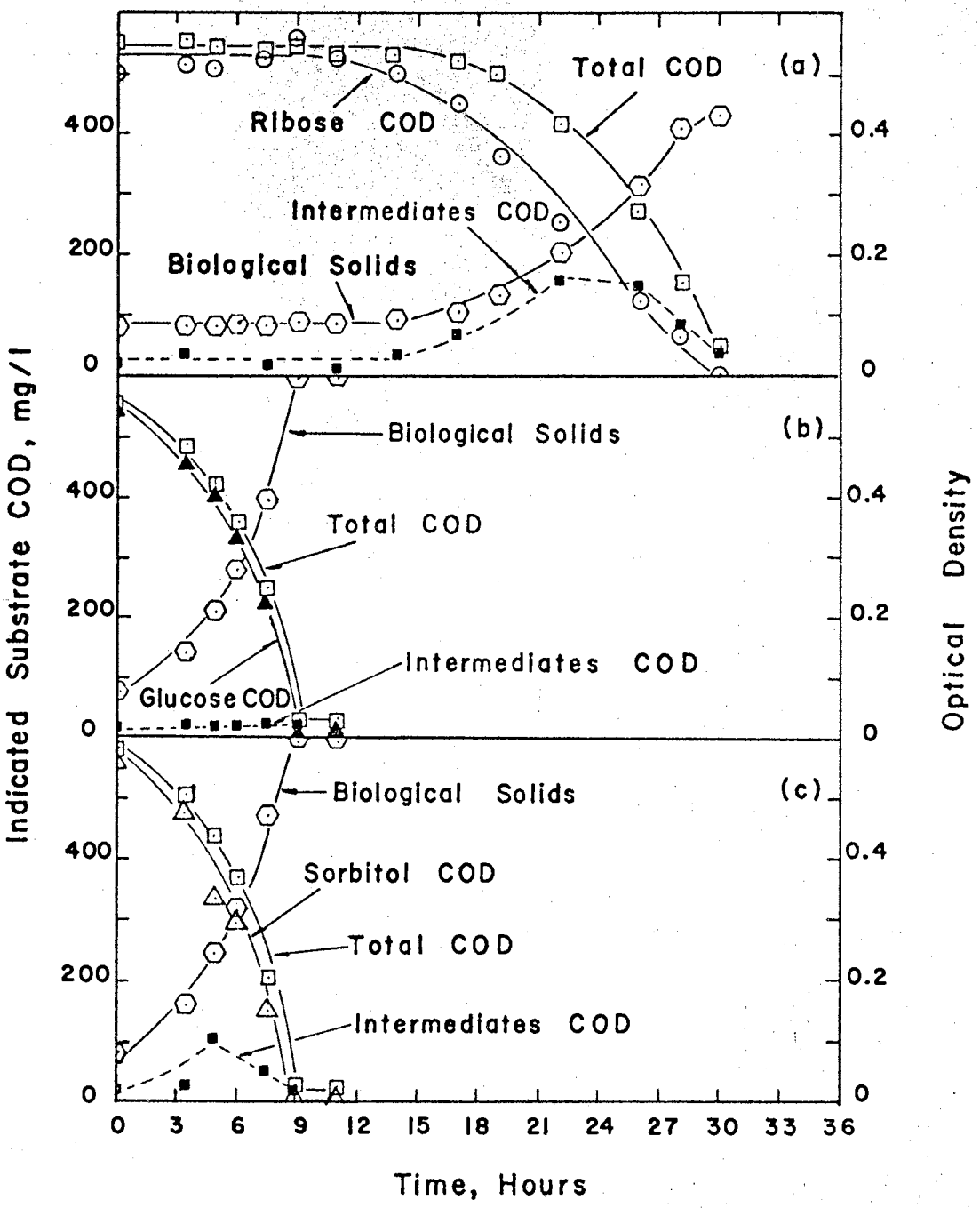

Figure 13 - System performance in the control units of (a) ribose, (b) glucose, and (c) sorbitol; young cells acclimated to sorbitol.

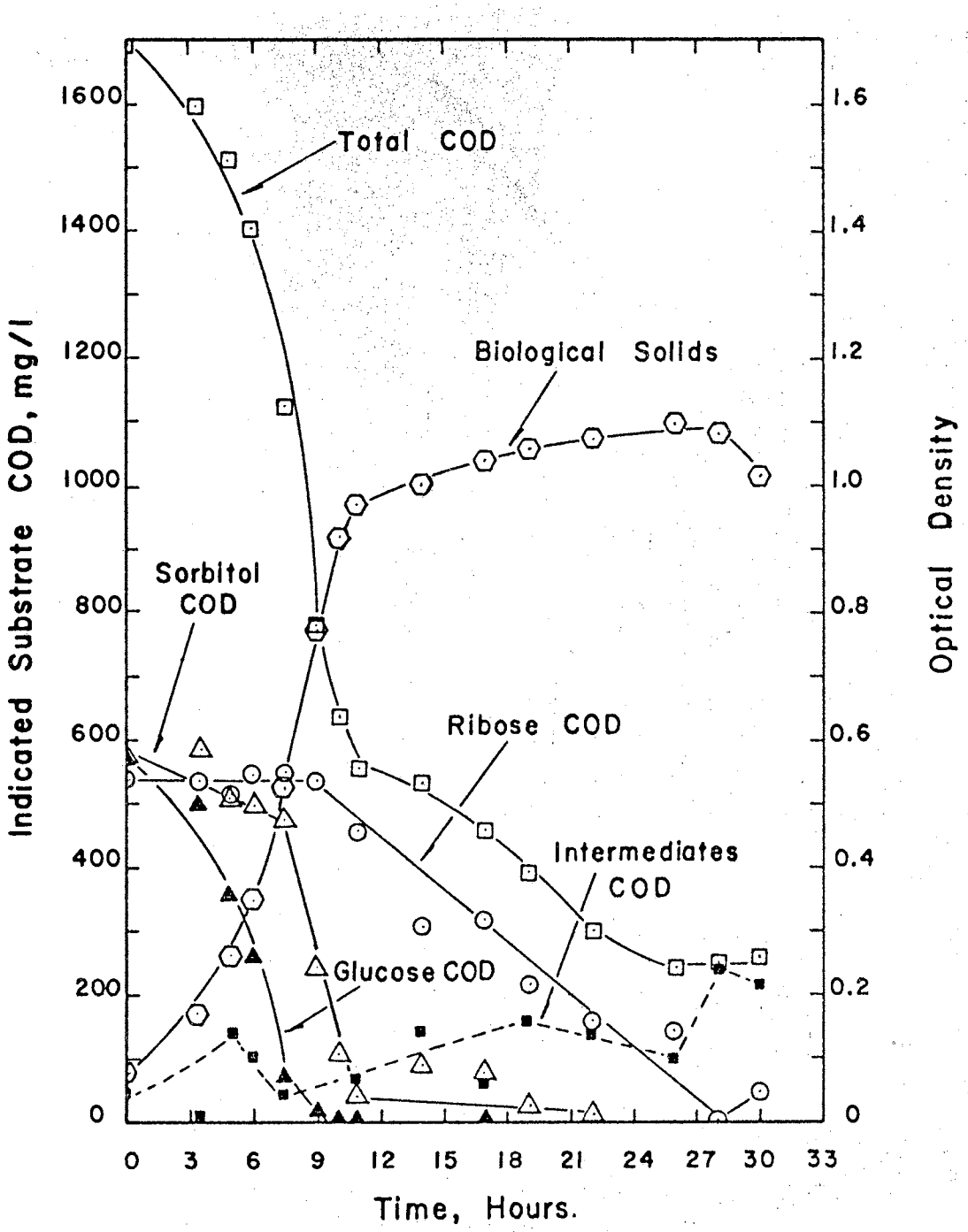

Figure 14 - System performance in the combined unit of glucose, sorbitol, and ribose; young cells acclimated to sorbitol.

of glucose and sorbitol more cells capable of metabolizing ribose were produced. The three substrates were removed sequentially in the order: glucose > sorbitol > ribose.

c. Ribose-acclimated Cells

The results for the control systems with cells acclimated to ribose are presented in Figure 15. It can be seen that the ribose-grown cells required a rather long acclimation period to sorbitol. Although the cells had been previously acclimated, they grew on ribose rather slowly (RT = 18.0 hrs) as compared to the growth on glucose (RT = 11 hrs). Semilogarithmic plots of OD versus time revealed no lag on ribose, but the logarithmic growth rate constant was significantly lower on ribose than on glucose, and slightly lower on ribose than sorbitol. Hence, more than 23 hours was required to consume 500 mg/l of ribose. Based upon growth rate and intermediates accumulation, the results for the control units do not clearly show the relative potential for interference between ribose and sorbitol. In any case, glucose possessed a rather high potential to interfere with removal of the other two carbon sources.

As in the previous two experiments, sequential removal of these three substrates was observed for the ribose-acclimated cells (Figure 16). However, the sequence of removal in this system (glucose > ribose > sorbitol) was different from that shown in Figures 12 and 14 (glucose > sorbitol > ribose). Comparing the glucose removal rates in the combined system (RT = 10 hrs) with that in the control (RT = 11 hrs), it is seen that glucose removal was only slightly enhanced in the presence of the other two substrates. The growth in the combined system (RT = 10.5 hrs) also was slightly faster than that in the glucose

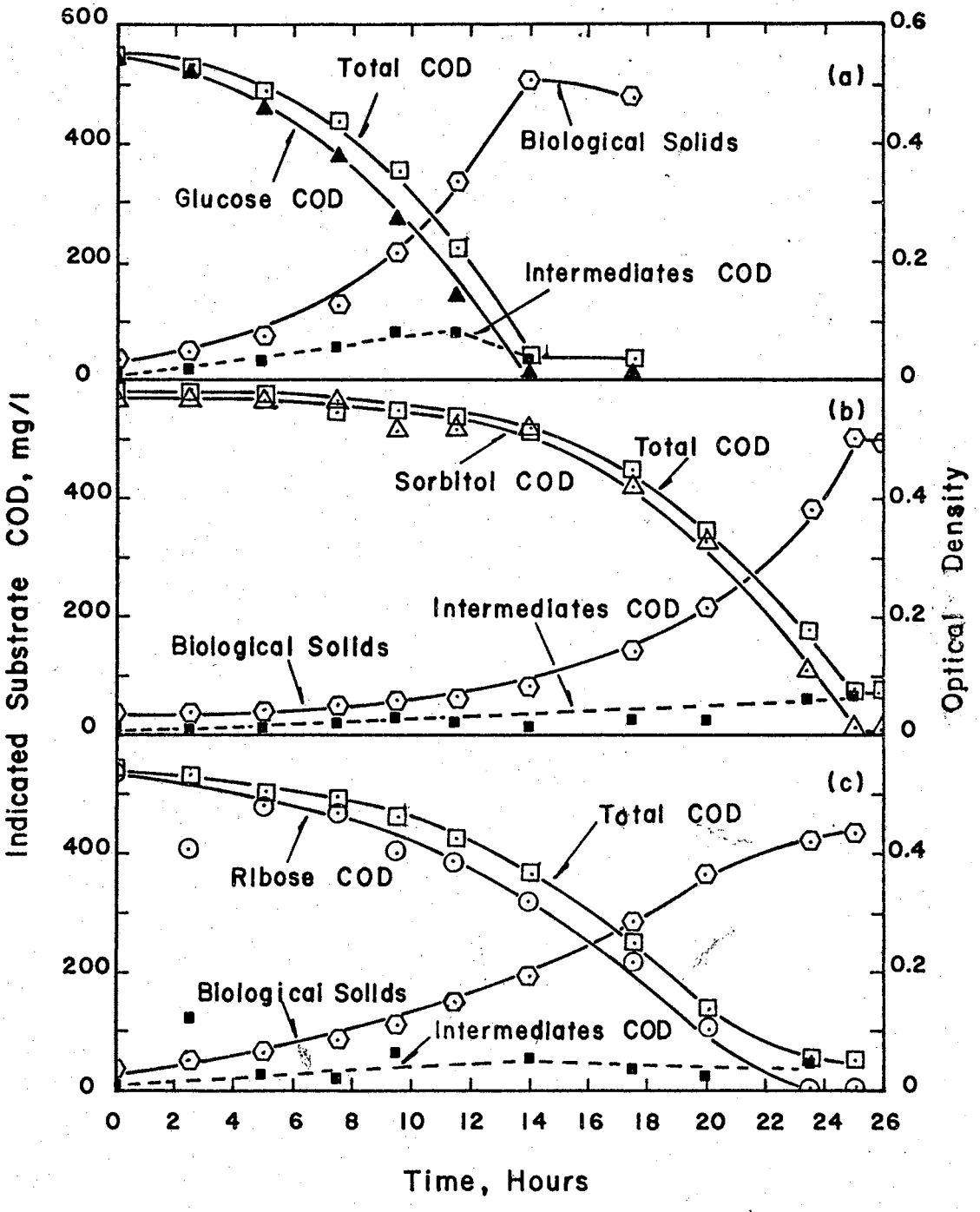

Figure 15 - System performance in the control units of (a) glucose, (b) sorbitol, and (c) ribose; young cells acclimated to ribose.

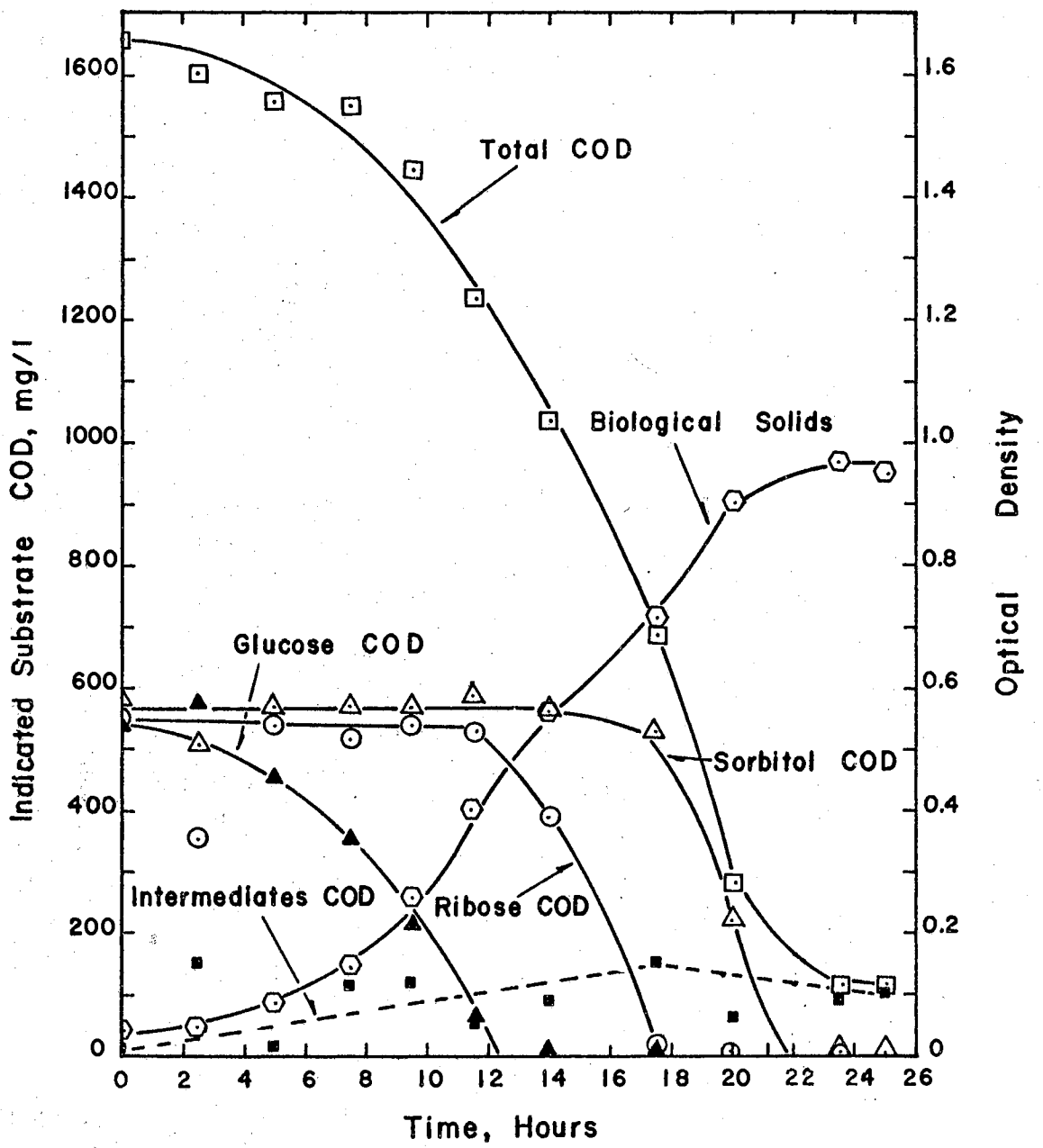

Figure 16 - System performance in the combined unit of glucose, ribose, and sorbitol; young cells acclimated to ribose.

control. The removal of ribose did not start until the glucose had been exhausted, indicating that ribose enzymes were inhibited by glucose. After release from glucose blockage, ribose was removed much faster. Only 4.7 hrs was required to reach the reference point as compared with 17.4 hrs in the ribose control. This observation suggests that the synthesis of ribose-utilizing enzymes proceeded during glucose metabolism. The finding that glucose did not completely repress the synthesis of ribose enzymes was also observed in Figures 12 and 14.

The synthesis of the sorbitol enzyme system was prolonged 10 hours in the combined reactor as compared with the lag period in the sorbitol control. This repression of enzyme synthesis can be attributed jointly to the presence of glucose, ribose, and the intermediates accumulated during glucose and ribose metabolism. From the results of Figures 12 and 14, it was seen that glucose exerted a repression on sorbitol enzyme synthesis to a limited extent. Thus it is reasonable to propose that ribose and the accumulated intermediates played an important role in repressing the synthesis of the sorbitol enzyme system.

From the results presented thus far, it is apparent that the substrate removal sequence which occurred in this experiment was probably caused by glucose inhibition of the ribose enzyme system and catabolite repression of the sorbitol enzyme system.

C. Studies on Substrate Removal in a Mixture of Glucose, Sorbitol, and Xylose by a Heterogeneous Population (Continuous Flow Reactor)

Because of the cost of ribose, continuous flow reactor studies using the glucose-sorbitol-ribose system were not undertaken. However, it was desirable to study the hexose and sugar alcohol in combination with an aldo-pentose, and xylose was selected to replace ribose. The

metabolic responses during continuous culture at various dilution rates are shown in Figure 17. The synthetic waste contained 500 mg/l of each carbon source. The reactor was inoculated with an acclimated cell suspension of milky appearance, and operated at a dilution rate of 1/24 hr^{-1}. Glucose, xylose, and sorbitol were used readily by this population. Within 1.5 days of operation at this dilution rate, the biological solids rose from 180 mg/l to 410 mg/l and only traces of xylose and sorbitol were detected in the effluent. An extremely low cell yield of 27 per cent was found at this period. Thereafter the mixed liquor started to develop a light greenish hue. Sorbitol leakage of 120 mg/l was detected at 2.9 days. It is rather difficult to explain the rapid biological solids accumulation which occurred from 2.5 to 5.5 days. During this period there was only a slight decrease (about 200 mg/l) in total COD concentration, but a significant increase in solids concentration (910 mg/l) was observed. While there was a decided change in predominating species (as judged by the color change), the high solids level attained may not be entirely due to an increase in cell yield from 27.2 to 80 per cent, but could have been due, at least in part, to an operational problem sometimes encountered in dealing with heterogeneous populations. At times during this experiment, heavy cell aggregates were formed which prevented complete mixing in the reactor. During the rapid rise in solids concentration, the optical density in the reactor was considerably higher than in the effluent, e.g., at 4.5 days, 0.903 versus 0.694. At this time the airflow rate was increased from 5000 to 7000 ml/min in order to provide better mixing of the flocculated solids. The solids concentration leveled off at slightly under 1200 mg/l, and the reactor and effluent OD values were 1.398 and 1.222,

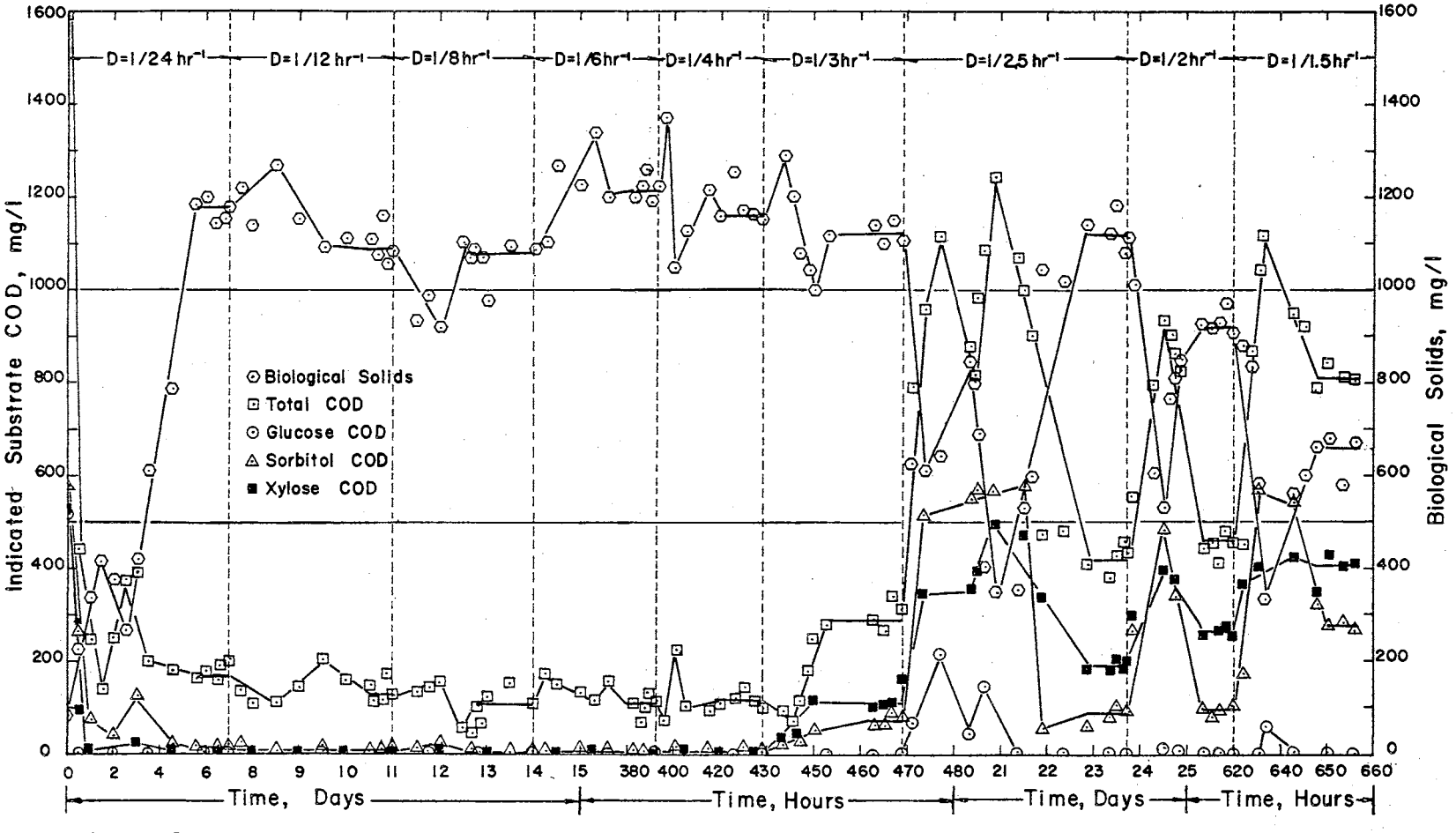

Figure 17 - System performance in the continuous flow activated sludge unit growing on glucose, sorbitol, and xylose at various dilution rates.

respectively. The system was more nearly completely mixed, and the biological solids concentration did appear to attain a relatively steady level. Data taken during this period (day 5.5 to 7.0) was adjudged to represent the "steady-state" behavior of the system at D = 1/24 hr^{-1}.

The dilution rate was next increased two-fold (from 1/24 to 1/12 hr^{-1}). It is seen that the hydraulic shock loading was not severe enough to cause a disruption of substrate removal efficiency. However, within 1.5 days after shifting the dilution rate, the biological solids concentration rose to 1270 mg/l and the COD decreased to 110 mg/l. It was noted that the shock gradually brought about more complete mixing in the reactor and the cells were more dispersed; the OD in the reactor and effluent were essentially the same. Although 200 mg/l COD was detected in the effluent at 9.5 days, only traces of sorbitol and essentially no xylose and glucose were found throughout operation at this flow rate. The unit was run for a total of 4 days (8 detention periods) and it is seen that both biological solids and effluent COD attained relatively steady levels (1090 mg/l and 130 mg/l, respectively). The cell yield value observed at "steady-state" was 72 per cent, while the efficiency of substrate removal was 92 per cent. The rather high cell yield value must be attributed to cell retention in the reactor.

It is seen that there was no significant system disruption during the experimental period of operation at 1/8 hr^{-1} dilution rate.

The flow was next adjusted to provide a dilution rate of 1/6 hr^{-1}. It is seen that after increasing the growth rate, there was a significant increase in cell concentration. During this time there was no severe disruption of substrate removal; however, the effluent COD rose slightly. After 50 hours, the effluent COD returned to its former

level (100 mg/l). At the new "steady-state," the biological solids concentration (approximately 1210 mg/l) attained somewhat higher levels than those observed during operation at the lower dilution rates. The steady-state cell yield at this dilution rate was determined to be 79 per cent. The optical density in the reactor was 1.523 and in the effluent 1.347; thus the unit cannot be said to have been precisely competely mixed.

The continuous flow unit was maintained at a dilution rate of 1/4 hr^{-1} for the next three days. In an attempt to measure the actual solids concentration of the system, i.e., the reactor, the samples at 1/4 hr^{-1} and other higher dilution rates were taken directly from the reactor. Four hours after shifting the flow rate, it was noted that the unit was not completely mixed (OD_r = 1.280 versus OD_e = 0.964). In an effort to help alleviate this situation, the floc was broken up in a Waring blender. The blending did have the desired effect in providing more complete mixing, since the flocculated organisms were quite dispersed (for example, OD in the reactor = 1.083 versus OD in the effluent = 1.034 at 401 hours).

After the unit had attained approximately steady-state at the 1/4 hr^{-1} dilution rate, the flow rate was changed to yield a dilution rate of 1/3 hr^{-1}. The efficiency of the system was disrupted severely. Within 21 hours (7 detention times), the biological solids concentration decreased from 1160 mg/l to 900 mg/l. The filtrate COD is also seen to have increased from 120 mg/l to 250 mg/l. The maximum substrate leakages during this period amounted to 120 mg/l of xylose COD and 60 mg/l of sorbitol COD. Slightly more than 8 detention periods were required before the system attained an approximate steady-state

condition. The reactor was adjudged to be completely mixed (OD_r = 1.204 versus Od_e = 1.171 at 469 hours). The approximate values of the system parameters in the steady-state condition were: 1020 mg/l biological solids, 290 mg/l effluent COD, 110 mg/l xylose COD, 75 mg/l sorbitol COD; no glucose COD was detected. The efficiency of COD removal was approximately 82 per cent.

Very severe fluctuations in biological solids and COD were observed after shifting the dilution rate to 1/2.5 hr^{-1}. After the initial dilute-out of cells, the appearance of the sludge changed and large heavy floc developed. The rise in solids from hour 474 to 488 was due mainly to solids retention in the reactor. At 488 hrs the OD in the reactor was 0.886 compared to 0.495 in the effluent. The heavy floc did not remain in the system for long. There was an apparent change in predominance; the floc became more dispersed and solids dilute-out continued. During this period there was a severe leakage of COD, and the order of leakage, which in the previous steady-state had been xylose > sorbitol > glucose, changed to sorbitol > xylose > glucose. After approximately 90 hours of operation at this dilution rate, the system attained a relatively steady-state. The order of substrate leakage was again xylose > sorbitol > glucose. The cell yield was very high, 91.8 per cent. The reactor exhibited a fairly good degree of complete mixing (OD_r = 1.280 versus OD_e = 1.140).

Another severe disruption was observed when the dilution rate was changed to 1/2 hr^{-1}. The filtrate COD rose from a steady-state value of approximately 420 mg/l to a transient peak value of 930 mg/l at 17 hours (584 hours on the time scale and 8.5 detention times after the shock), and thereafter decreased to a new steady-state level of

approximately 460 mg/l. There was a significant change in biological solids level. The biological solids were "diluted out" from 1120 mg/l to the minimum concentration of 530 mg/l and then attained a "steady-state" value of approximately 920 mg/l. There was considerable leakage of substrate during the transient state; the maximum substrate concentrations in the effluent during this period amounted to 480 mg/l sorbitol COD and 390 mg/l xylose COD. About 40 hours (20 detention times) were required for the system to regain a condition of steady behavior; at this time the unit was adjudged to be completely mixed (OD_r = 0.969 versus OD_e = 0.921). The sludge yield during this steady-state period was 77.7 per cent. The steady-state parameters were: 920 mg/l biological solids, 460 mg/l effluent COD, 265 mg/l xylose COD, and 95 mg/l sorbitol COD. The order of leakage was the same as for the previous dilution rates (1/2.5 and 1/3 hr^{-1}).

The highest dilution rate applied was 1/1.5 hr^{-1}. In this case, the biological solids concentration decreased to 340 mg/l within 14 hours, while effluent COD increased to 1100 mg/l (approximately 60 mg/l glucose, 400 mg/l xylose, and 550 mg/l sorbitol). During this period the light brownish color of the mixed liquor disappeared completely, and thereafter a population consisting predominantly of light greenish filamentous organisms appeared. The reactor was not completely mixed; biological solids were being retained in the reactor. Although the system eventually approached an approximate steady condition with respect to biological solids values and effluent COD, the cell yield value, 79.5 per cent, was falsely high because of incomplete mixing, e.g., OD_r = 0.854 versus OD_e = 0.678 at 656 hours. The approximate "steady" values of biological solids (660 mg/l), effluent COD (810 mg/l)

and substrate leakages (270 mg/l sorbitol COD and 400 mg/l xylose COD) were used as the steady-state parameters at this dilution rate.

Figure 18 shows the steady-state levels of the various parameters taken from Figure 17 for each dilution rate. It is seen that all three carbon sources were utilized completely at dilution (growth) rates lower than 0.25 hr^{-1} (1/4 hr^{-1}). Glucose was the preferred carbon source and was removed even at a dilution rate as high as 1/1.5 hr^{-1} (0.667 hr^{-1}). The presence of glucose in the feed led to substantially less utilization of both sorbitol and xylose at dilution rates higher than 1/4 hr^{-1}. At a dilution rate of 1/1.5 hr^{-1}, the efficiency of sorbitol removal was reduced to 53 per cent, while only 25 per cent of the xylose was removed.

Even though at times there was some difficulty in maintaining a completely mixed condition, the results clearly indicate that the system began to undergo dilute-out at dilution rates exceeding 0.25 hr^{-1} and that the order of preferential substrate utilization, regardless of various changes in predominance which occurred, was glucose > sorbitol > xylose. It is interesting to note that the proportion of metabolic intermediates (i.e., COD not attributable to the sum of glucose, sorbitol and xylose) did not increase with increased dilution rate. Total intermediate COD ranged from 100 to 150 mg/l, and only a small portion of these intermediates was accounted for as pyruvate (10 mg/l) and acetic acid (10 mg/l). A somewhat higher amount could be accounted for as carbohydrate (anthrone-reactive material).

D. Studies on Substrate Removal in a Mixture of Glucose, Galactose and Sucrose by a Heterogeneous Population

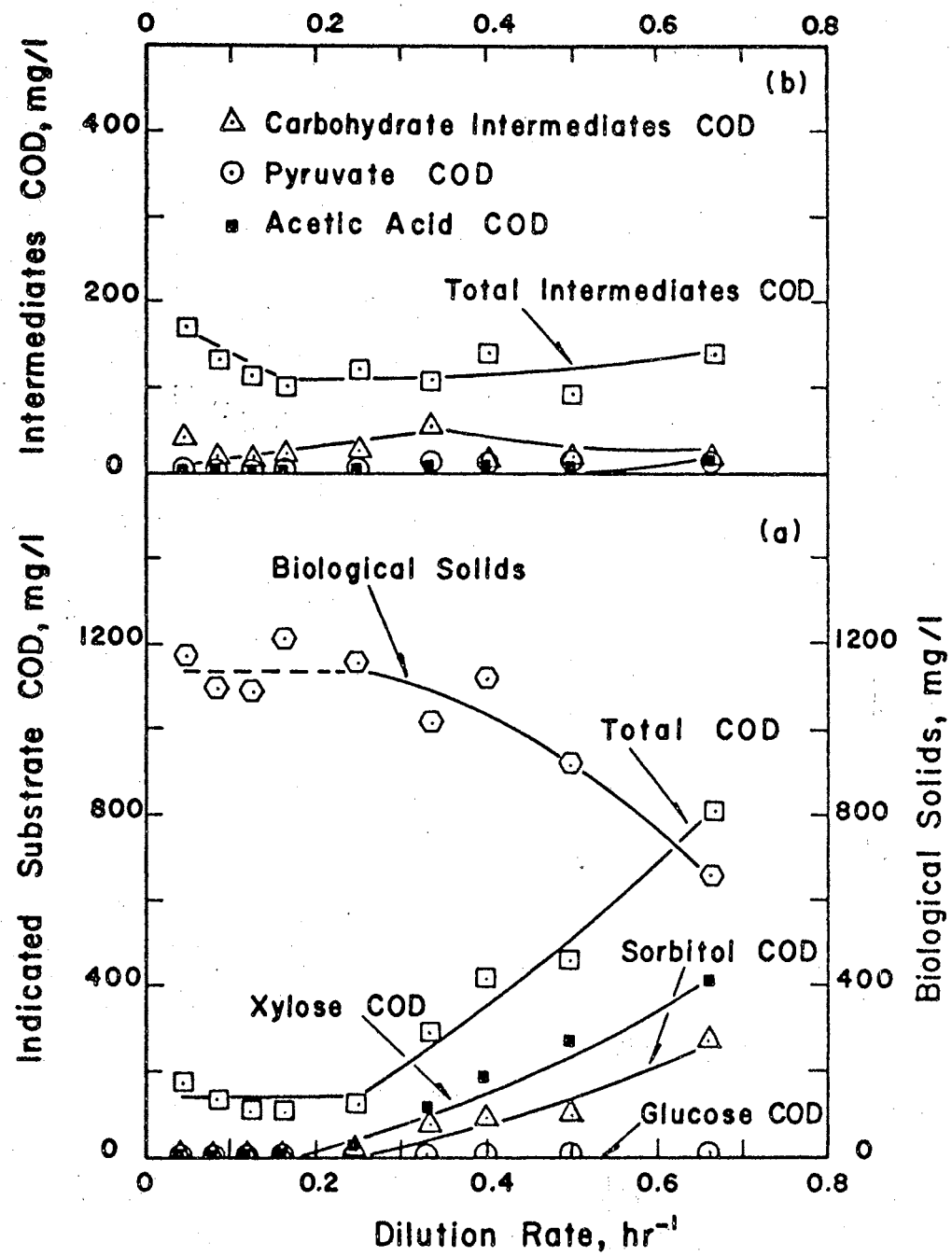

Figure 18 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, xylose, and sorbitol at various dilution rates; (b) intermediates accumulation.

I. Batch Experiments

a. Glucose-acclimated Cells

Results for the control units are shown in Figure 19. Cells grown on glucose acclimated rapidly to galactose and sucrose. Semilogarithmic plots of OD versus time indicated only a short lag period on these substrates. The cells grew on galactose rather slowly (RT = 16.5 hrs) as compared with the growth on glucose (RT = 10.9 hrs) or on sucrose (RT = 12.0 hrs). A somewhat greater amount of metabolic intermediates was accumulated from sucrose consumption (165 mg/l) than from glucose (115 mg/l) or galactose (110 mg/l).

Biological growth and substrate utilization in the combined glucose, sucrose and galactose system are given in Figure 20. A comparison of glucose removal rate in the combined system (RT = 9.0 hrs) with that in the control system (RT = 8.9 hrs) indicates that glucose removal was neither enhanced or retarded in the presence of the other two sugars. A comparison of the reference times for the removal of galactose and sucrose in the combined system (12 and 14.4 hrs) with those in the control systems (14.4 and 11.2 hrs) indicates that galactose removal was enhanced by the presence of other sugars whereas sucrose removal was significantly retarded. Because of this substrate interference, the sequence of removal in this mixed-substrate reactor was glucose > galactose > sucrose.

b. Galactose-acclimated Cells

Results for the control systems when cells acclimated to galactose

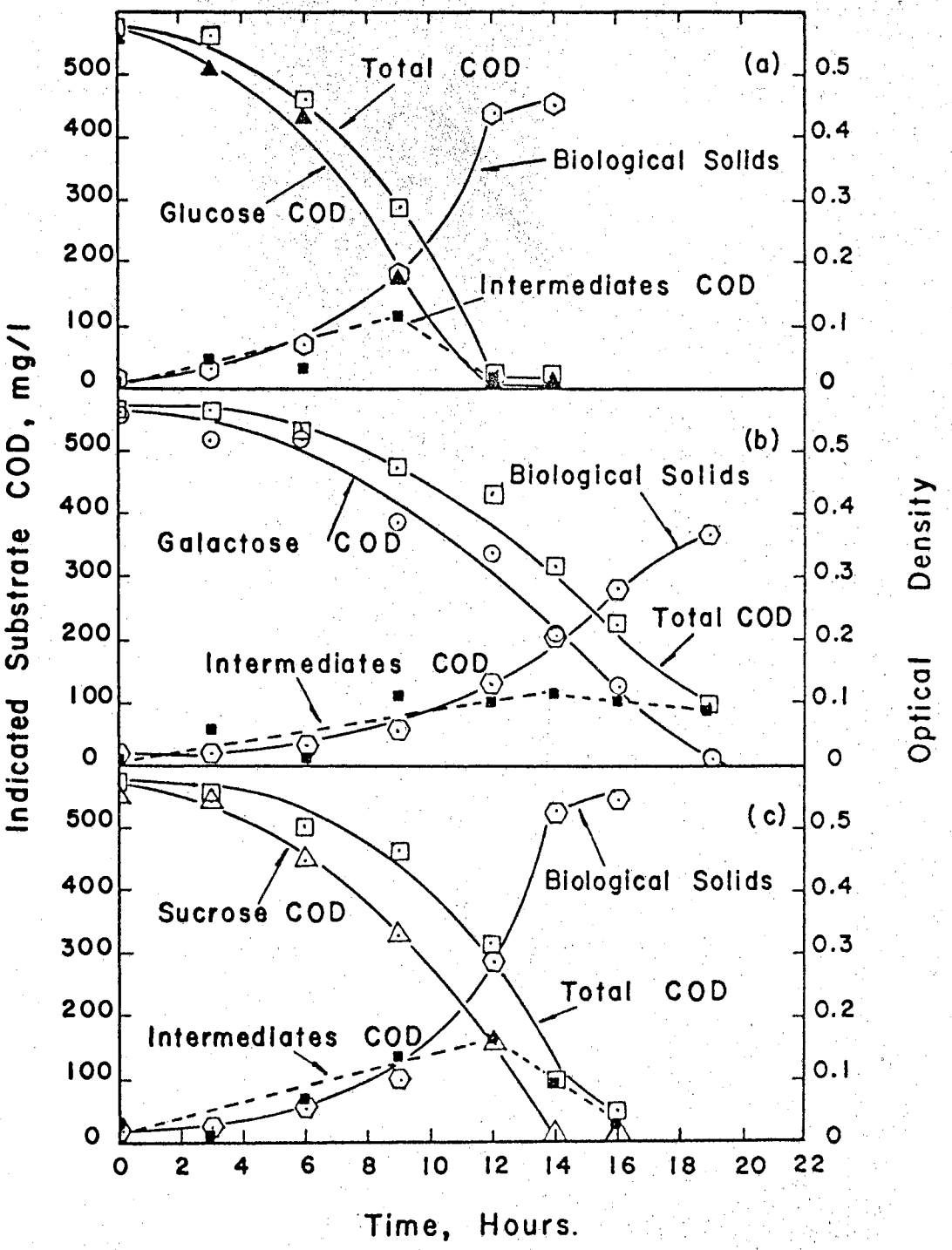

Figure 19 - System performance in the control units of (a) glucose, (b) galactose, and (c) sucrose; young cells acclimated to glucose.

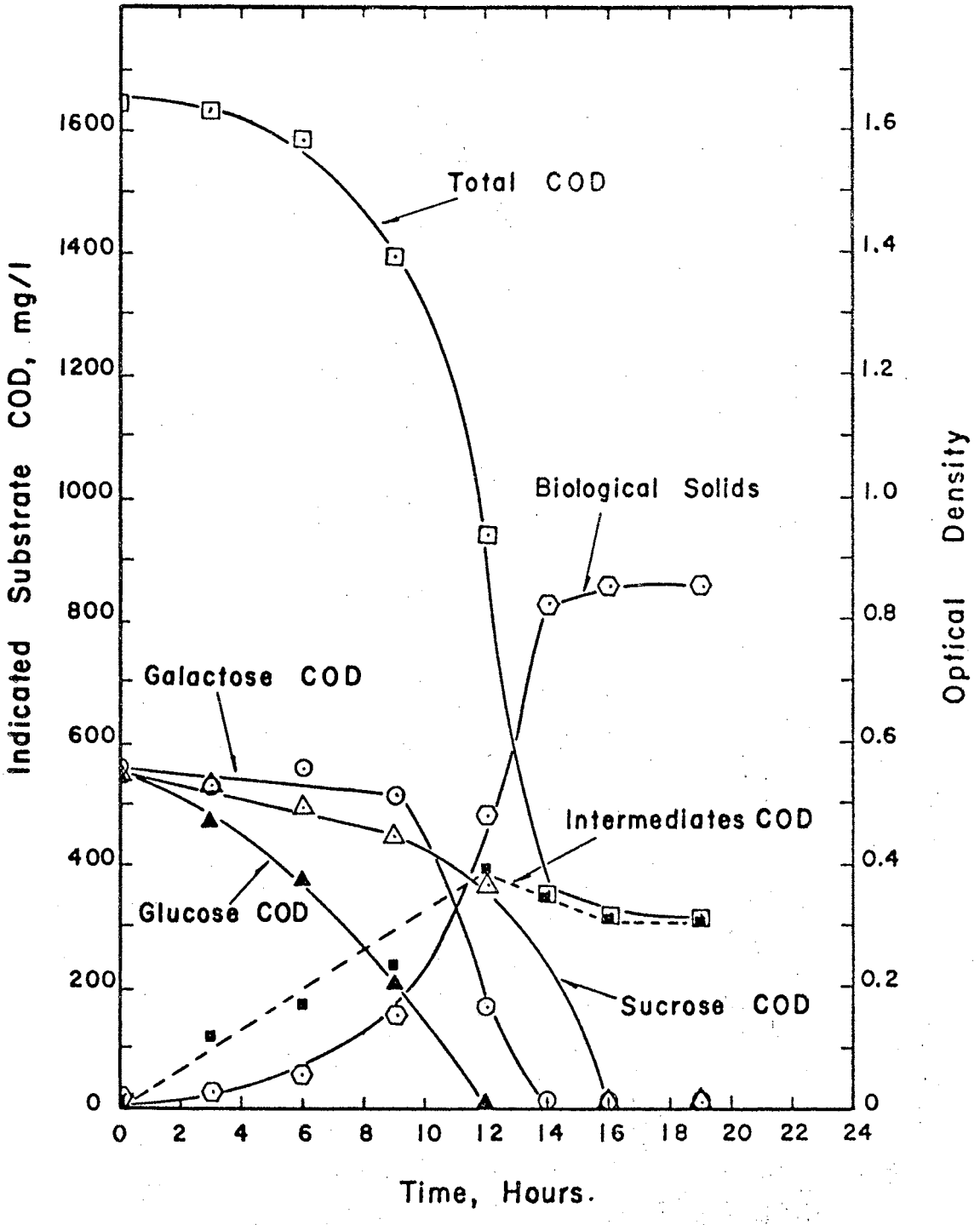

Figure 20 - System performance in the combined unit of glucose, galactose, and sucrose; young cells acclimated to glucose.

were employed are shown in Figure 21. The cells grew on glucose (RT = 4.2 hrs) and sucrose (RT = 4.6 hrs) more rapidly than on galactose (RT = 5.3 hrs). The intermediate COD curves show that more metabolic intermediates accumulated in the glucose reactor than in the other two units. These observations suggest that glucose exhibits a potential ability to interfere with the utilization of the other two sugars when these substrates are used as a combined carbon source.

When the sugars were used as combined carbon source (Figure 22) it is seen that glucose consumption was enhanced by about one hour (RT = 3.2 hrs in the combined system and 4.2 hrs in the control), while both utilization of galactose (from RT = 5.8 to 6.4 hrs) and sucrose (from RT = 5.1 to 6.2 hrs) were slightly retarded. The linear glucose removal indicated that glucose was being removed by preformed enzymes and that no additional synthesis of a glucose-degrading enzyme system occurred. It is interesting to note that the accumulation of metabolic intermediates amounted to 610 mg/l at 7 hours. Thus approximately 42 per cent of the substrate which was metabolized was accumulated in the medium in the form of compounds other than in the original sugars. The residual COD of this system, 300 mg/l, was about the same as that which remained in the system with glucose-acclimated cells (Figure 20). The linear removal of galactose (K_g = 21 mg/l/hr) while glucose was still present in the system indicates that glucose repressed the further formation of new galactose enzymes, but did not inhibit, at least not completely, the activities of existing enzymes. The facts that sucrose removal was not enhanced after glucose was removed and that galactose

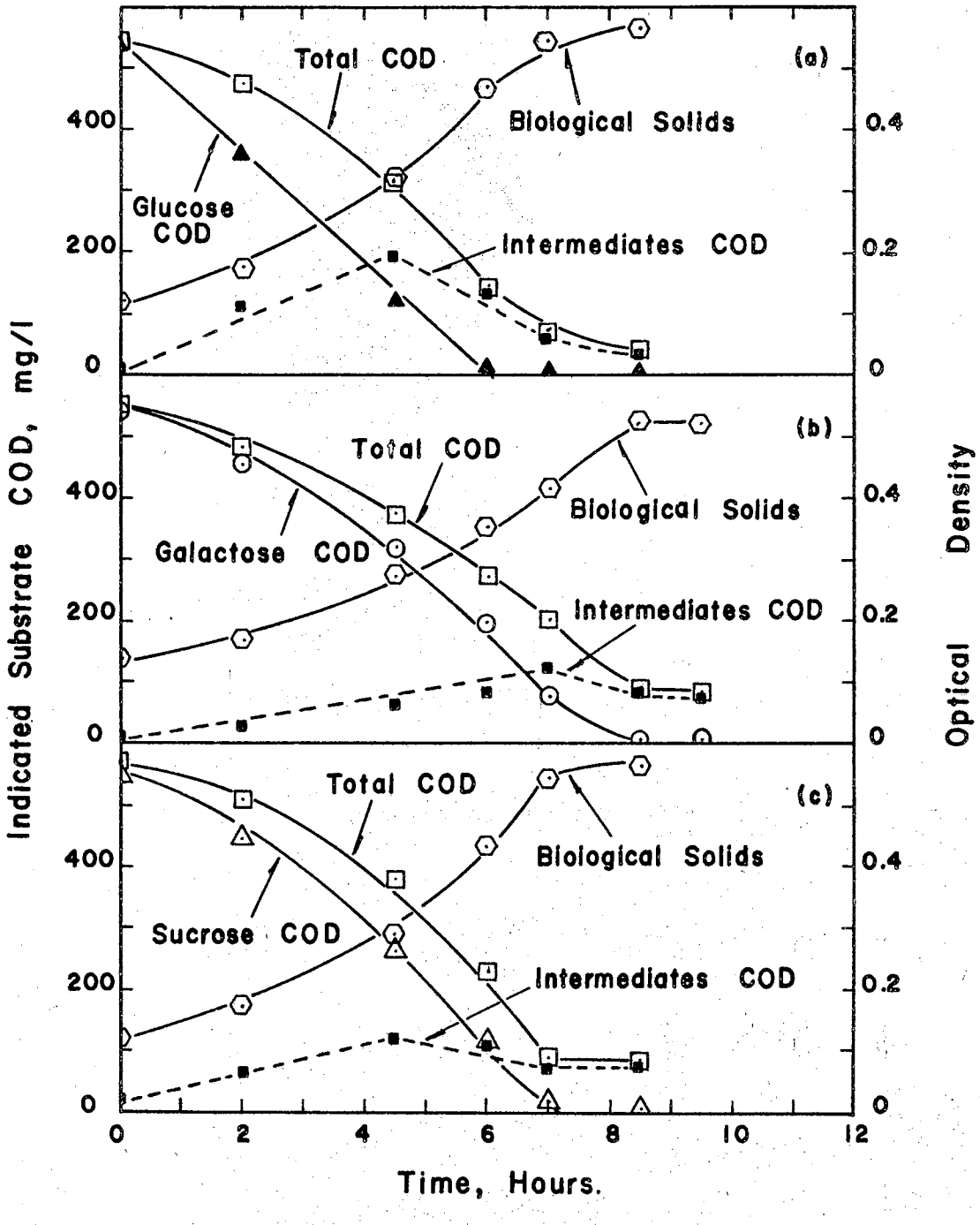

Figure 21 - System performance in the control units of (a) glucose, (b) galactose, and (c) sucrose; young cells acclimated to galactose.

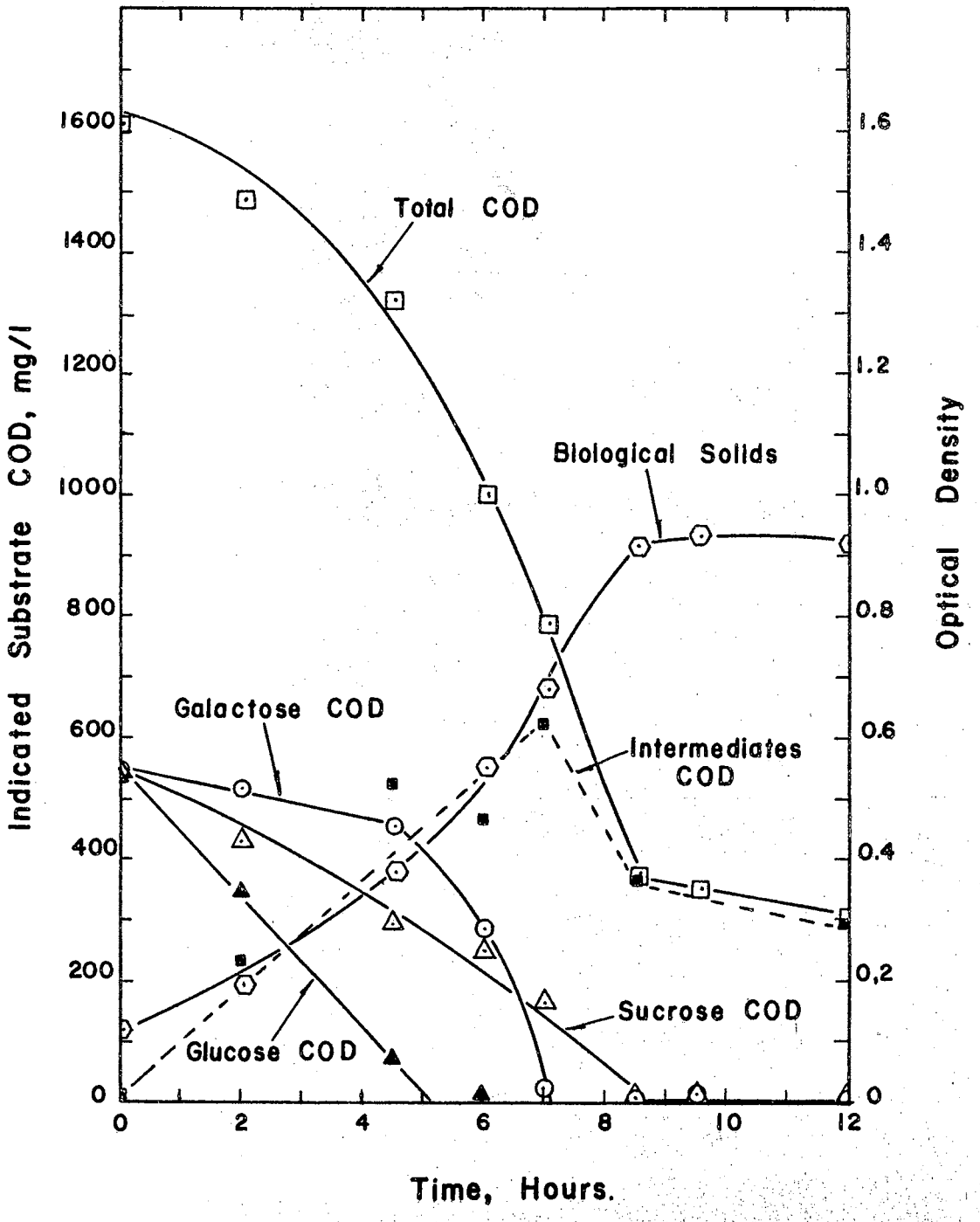

Figure 22 - System performance in the combined unit of glucose, galactose, and sucrose; young cells acclimated to galactose.

was exhausted earlier than sucrose indicate that sucrose removal might have been subject to interference by galactose. As with glucose-acclimated cells (Figure 20), the order of priority for substrate removal was glucose > galactose > sucrose.

c. Sucrose-acclimated Cells

The biological growth patterns which were exhibited when the sugars were used separately (Figure 23) indicate that the sucrose-acclimated cells grew on glucose (RT = 5.6 hrs) and sucrose (RT = 5.6 hrs) more rapidly than on galactose (RT = 8.6 hrs). Glucose metabolism produced a greater accumulation of metabolic intermediates (150 mg/l) than did sucrose (100 mg/l) or galactose (60 mg/l). Thus, the suggested relative potential for substrate interference is glucose > sucrose > galactose.

The sequence of substrate consumption for the combined system (Figure 24) was in accordance with the relative potential for interference as judged from the results of control reactors, i.e., glucose > sucrose > galactose. Comparison of glucose removal and biological growth in the combined system (RT = 5.6 and 5.4 hrs) with the glucose control (RT = 5.2 and 5.6 hrs) system indicates that the cells in the combined reactor grew slightly faster and did not remove glucose more rapidly than in the glucose control. Some galactose and sucrose were removed concurrently with glucose. The removal curves for galactose and sucrose show that 70 mg/l of galactose and 125 mg/l of sucrose were removed during the reference time for glucose consumption. Glucose blocked sucrose removal to a considerable extent. It is noted that about 330 mg/l of metabolic products had accumulated at the time of glucose exhaustion. Thereafter, the metabolism of galactose and sucrose

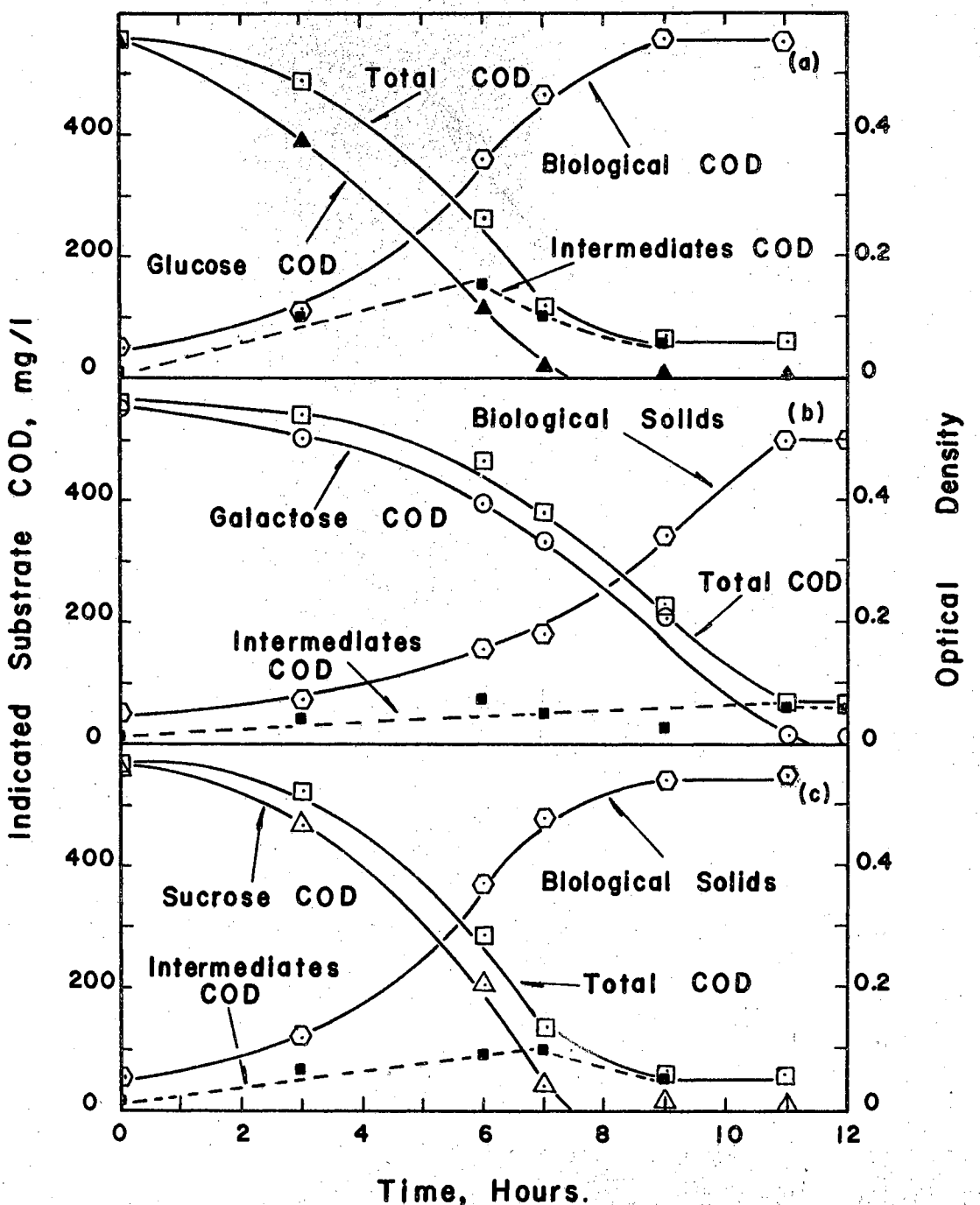

Figure 23 - System performance in the control units of (a) glucose, (b) galactose, and (c) sucrose; young cells acclimated to sucrose.

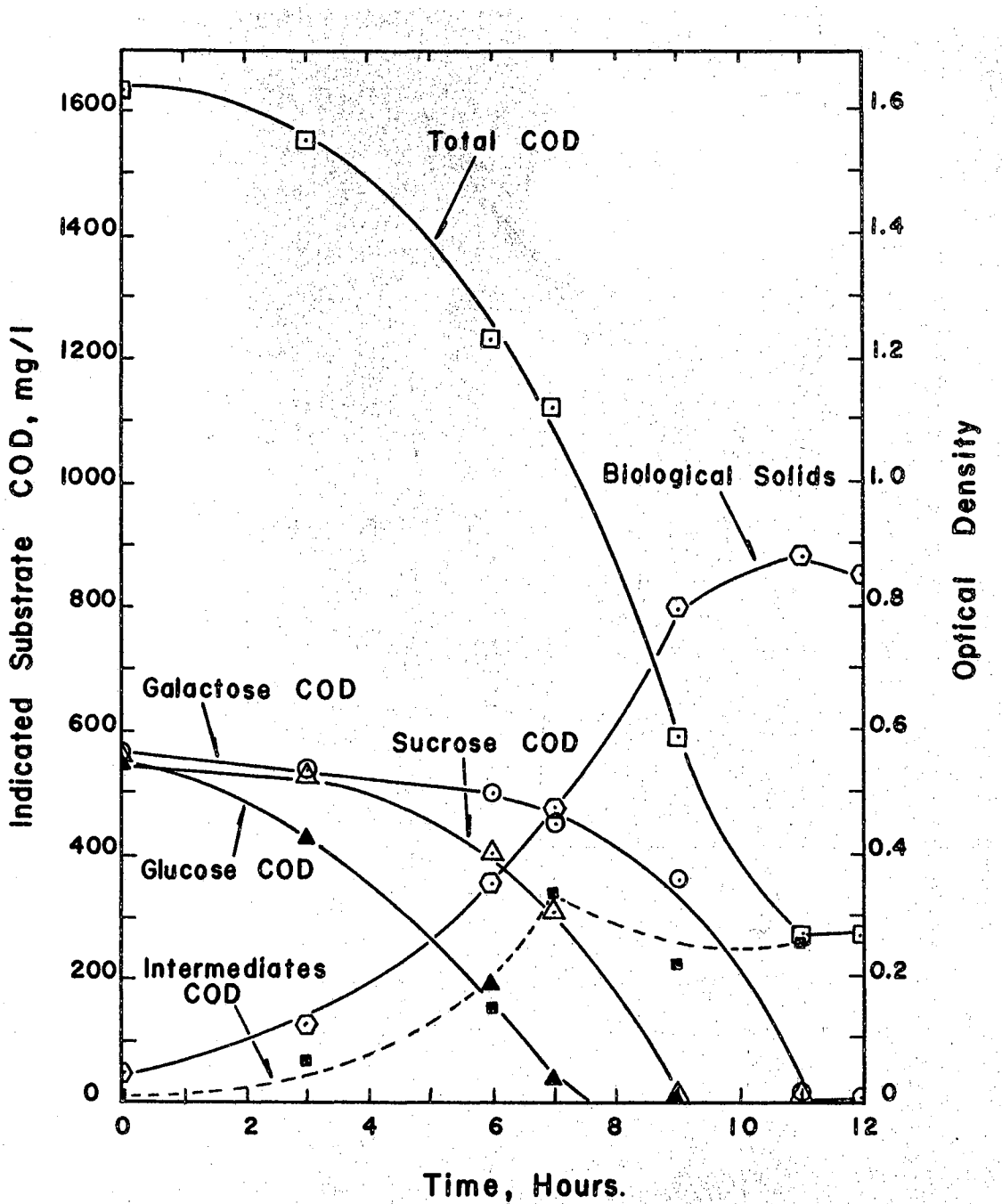

Figure 24 - System performance in the combined unit of glucose, galactose, and sucrose; young cells acclimated to sucrose.

did not increase the accumulation of intermediates. The system efficiency for the joint carbon source system was determined to be 85 per cent (compared to 82 per cent for the other combined systems). It is interesting to note that the order of substrate consumption in this system (glucose > sucrose > galactose) was quite different from that of the previous two systems (glucose > galactose > sucrose).

II. Continuous Flow Experiments

Growth patterns on the combined glucose-galactose-sucrose substrate in the continuous flow reactor were studied at seven dilution rates (see Figure 25). There was no significant disruption of substrate removal efficiency during the period of operation at dilution rates from 1/24 hr^{-1} to 1/4 hr^{-1}. The COD removal efficiency varied from 91.9 to 17.5 per cent. The cell yields were determined to be 40.1, 47.0, 51.3, 44.9, and 51 per cent, respectively.

When the dilution rate was changed from 1/4 to 1/3 hr^{-1}, a severe disruption of substrate removal efficiency ensued, a well-defined transient pattern was observed, and then there was a recovery and return to a steady-state (72 hours after the shift in dilution rate). Within 36 hours after the change in dilution rate (12 detention times) the biological solids level dropped from 760 mg/l to a minimum value of 80 mg/l, and the effluent COD increased from 110 mg/l to a transient peak value of 1540 mg/l. At the severest disruption period (546 hrs on the scale) more than 500 mg/l of galactose COD, about 300 mg/l of sucrose COD, and 250 mg/l of glucose COD were found in the reactor effluent. Thirty-six hours later, a new steady-state was approached but there was a considerable leakage of COD. The effluent COD was approximately 630 mg/l (42.5 per cent efficiency) and the biological

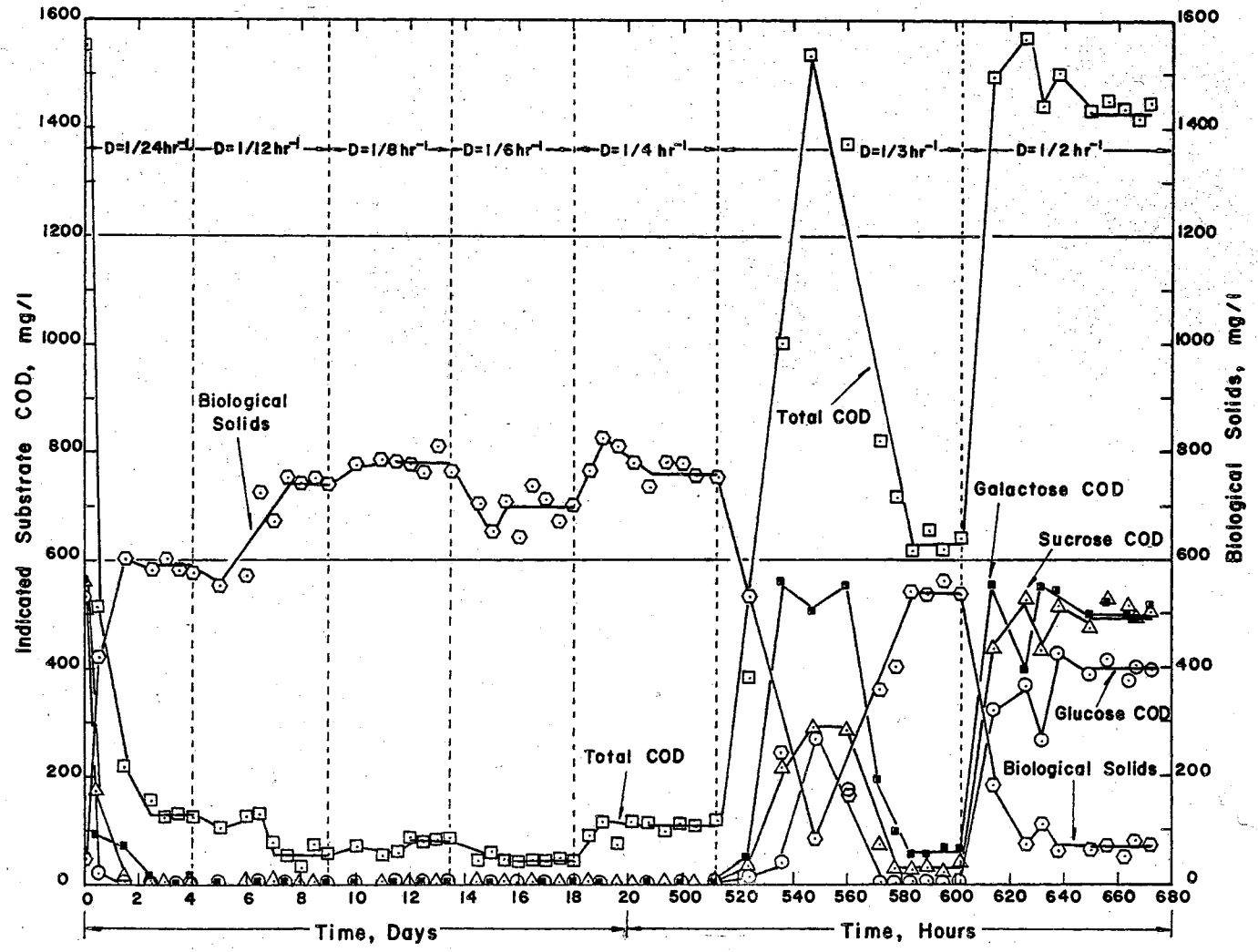

Figure 25 - System performance in the continuous flow activated sludge unit growing on glucose, galactose, and sucrose at various dilution rates.

solids level in the reactor was approximately 540 mg/l. Thus it appeared that the system was beginning the dilute-out phase. It is important to note that throughout this series of experiments, there was no problem with complete mixing in regard to biological solids. In the effluent COD, about 60 mg/l was identified as galactose and 30 mg/l as sucrose. Although the solids concentration level was much lower than the previous values, the steady-state cell yield (55.7 per cent) was higher than at any other dilution rate.

Nearly complete dilute-out was observed when the dilution rate was increased to 1/2 hr^{-1}. Within 24 hours (12 detention times), the biological solids level decreased to 70 mg/l and there was a corresponding effluent COD of 1570 mg/l. There was considerable fluctuation in the concentration of glucose, galactose, and sucrose during the transient period. However, the sugar concentration appeared to reach a new steady-state level (400 mg/l of glucose, 490 mg/l sucrose, and 500 mg/l galactose) at the end of the experiment, i.e., sucrose and galactose were not utilized. The steady-state solids concentration was 70 mg/l, while the effluent COD was 1430 mg/l. The steady-state system efficiency was only 10.6 per cent, and the cell yield was 41.2 per cent.

The average steady-state parameters and intermediate production at various detention times are presented in Figure 26. It is seen that at dilution (growth) rates lower than 1/4 hr^{-1}, all three sugars were removed completely. At a dilution rate of 1/3 hr^{-1}, small amounts of galactose and sucrose began to leak from the reactor. It is interesting to note that substantial excretion of metabolic intermediates (540 mg/l) was also observed at this dilution rate. The intermediates were found to consist of 160 mg/l carbohydrate, 90 mg/l pyruvate, 50 mg/l

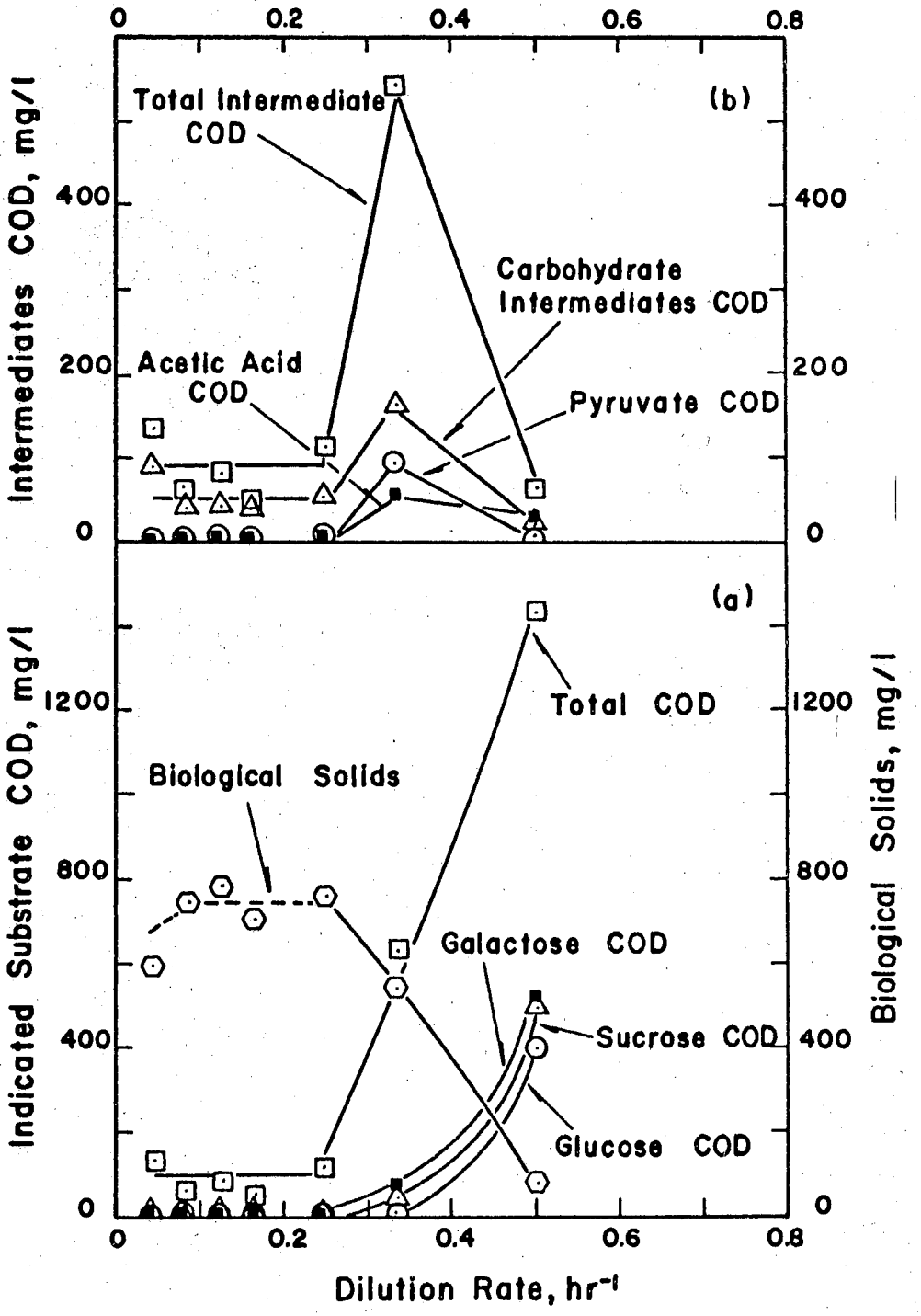

Figure 26 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, galactose, and sucrose at various dilution rates; (b) intermediates accumulation.

acetate and 240 mg/l unidentified components. When dilution rate was increased by 1/2 hr^{-1} most of the sugars (94 per cent of the galactose, 93 per cent of the sucrose and 75 per cent of the glucose) remained unutilized and the amount of intermediates decreased to about 50 mg/l.

E. Studies on Substrate Removal in a Mixture of Glucose, Xylose and Sucrose by a Heterogeneous Microbial Population

I. Batch Experiments

a. Glucose-acclimated Cells

The responses for the single substrate units are presented in Figure 27. A long lag (more than 10 hours) was observed in xylose metabolism, indicating that enzymes responsible for the metabolism of xylose were not constitutive in glucose-acclimated sludge. The growth curves show that glucose (RT = 8.2 hrs) and sucrose (RT = 11.2 hrs) supported much faster growth than did xylose (RT = 30.4 hrs). This observation was borne out by the semilogarithmic plots which were made to determine values of μ. It is seen that a slightly higher amount of metabolic intermediates accumulated in the xylose system than in the sucrose system. Based on rate of growth, accumulation of metabolic intermediates and required acclimation, the following order of relative potential for substrate interference or order of removal can be predicted for these three sugars: glucose > sucrose > xylose.

In the combined system (Figure (28), there is a clear indication that the order of removal was glucose > sucrose > xylose. This result is coincident with the potential sequence of substrate interference estimated from the results of control units. It is discerned that catabolism of glucose was not affected by the presence of the other sugars (RT = 7.2 hrs in the combined system versus RT = 7.0 hrs) in the control.) Sucrose metabolism was slightly retarded by glucose catabolism (RT =

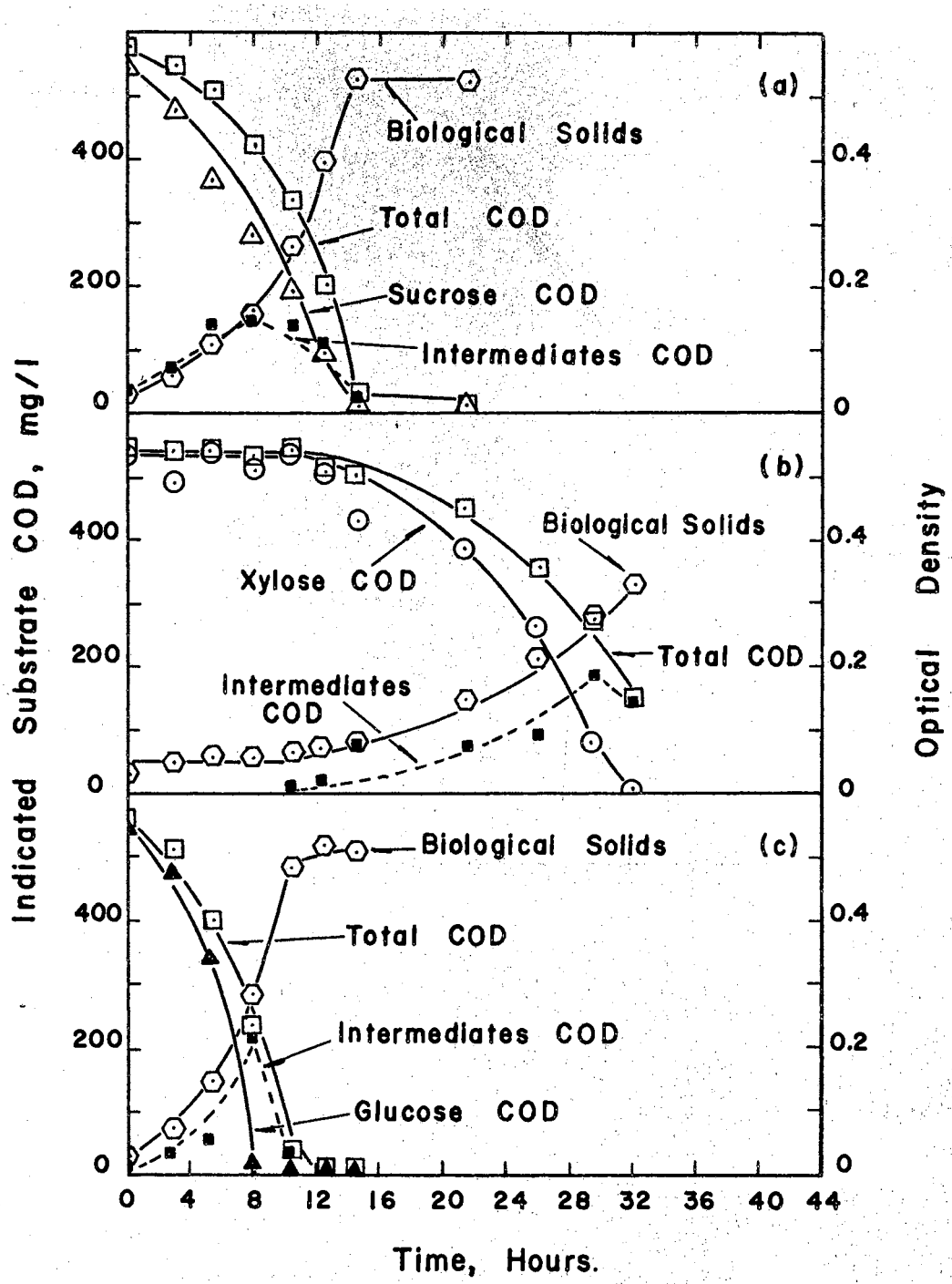

Figure 27 - System performance in the control units of (a) sucrose, (b) xylose, and (c) glucose; young cells acclimated to glucose.

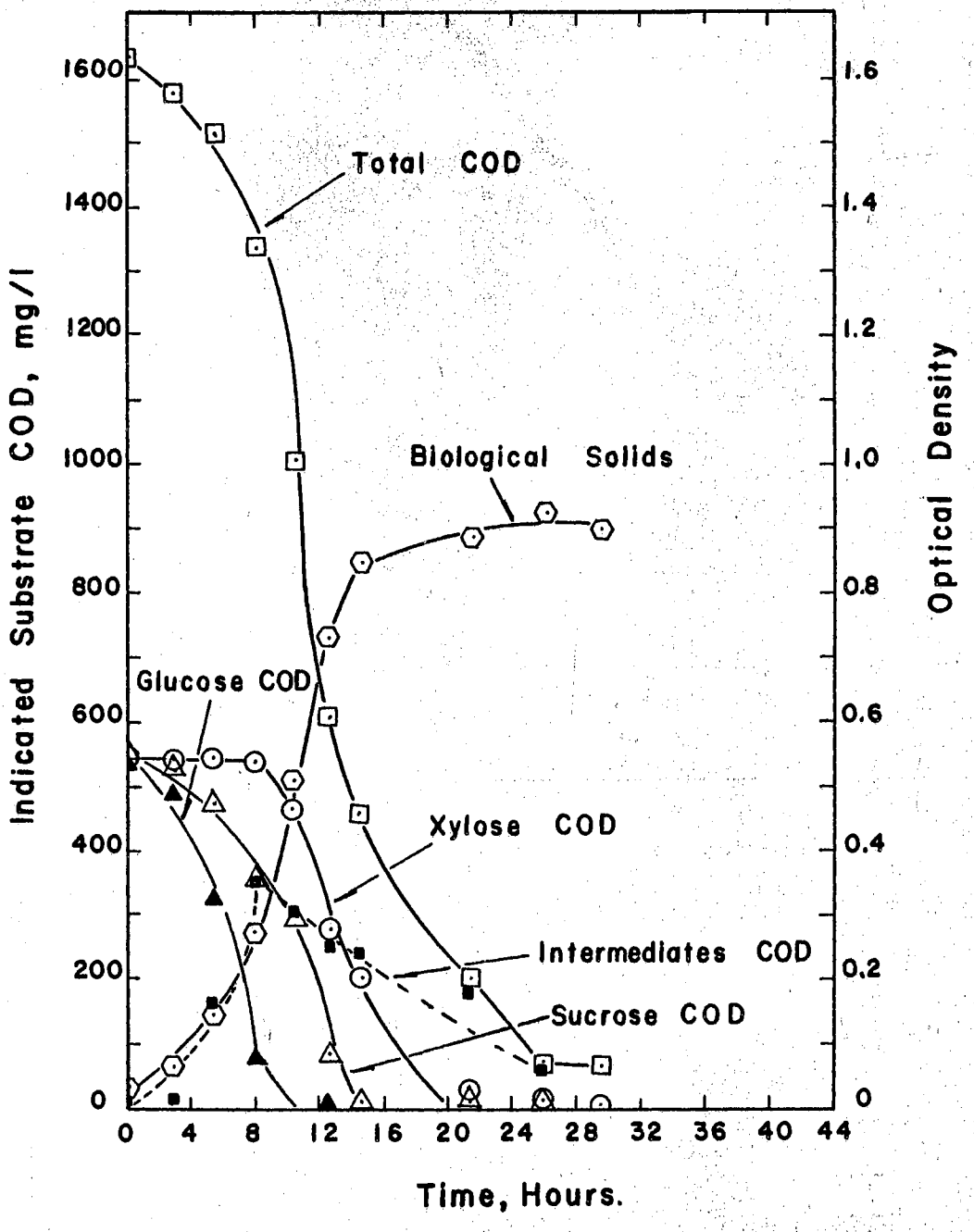

Figure 28 - System performance in the combined unit of glucose, xylose, and sucrose; young cells acclimated to glucose.

12.2 hrs for combined and 10.2 hrs for control system). Comparison of the lag period required for xylose removal in the combined system (about 8 hrs) with that in the control (more than 10 hrs) suggests that the synthesis of xylose-degrading enzymes was greatly enhanced by metabolism of the other two sugars. However, it should also be noted that a great number of cells were present in the combined system. It is seen that a considerable amount of metabolic intermediates was excreted into the medium during the course of mixed-substrate metabolism. Since the peak accumulation of intermediates (about 350 mg/l) occurred when 85 per cent of the glucose had been consumed, it may be concluded that the excretion of intermediates was due largely to the metabolism of glucose. Semilogarithmic plots of OD versus time and total COD removal versus time revealed that diphasic growth corresponded to the two-phase total COD removal.

b. Xylose-acclimated Cells

The results for the three control reactors (Figure 29) indicate that the xylose-grown cells grew on xylose rather slowly (RT = 9.5 hrs) as compared to the growth on glucose (RT = 6.2 hrs) and sucrose (RT = 5.8 hrs). The logarithmic growth rate constants were $\mu_x = 0.195\ hr^{-1}$, $\mu_g = 0.278\ hr^{-1}$, and $\mu_s = 0.314\ hr^{-1}$. It is interesting to note that the relative amounts of intermediates which accumulated during the metabolism of these three sugars was in the order: sucrose > glucose > xylose. Therefore, it seems reasonable to expect that sucrose and glucose possess a potential to interfere with the catabolism of xylose, if these three sugars are mixed together in a reactor.

The response in the combined system is shown in Figure 30. Comparing glucose removal in the combined system (RT = 6.2 hrs) with that

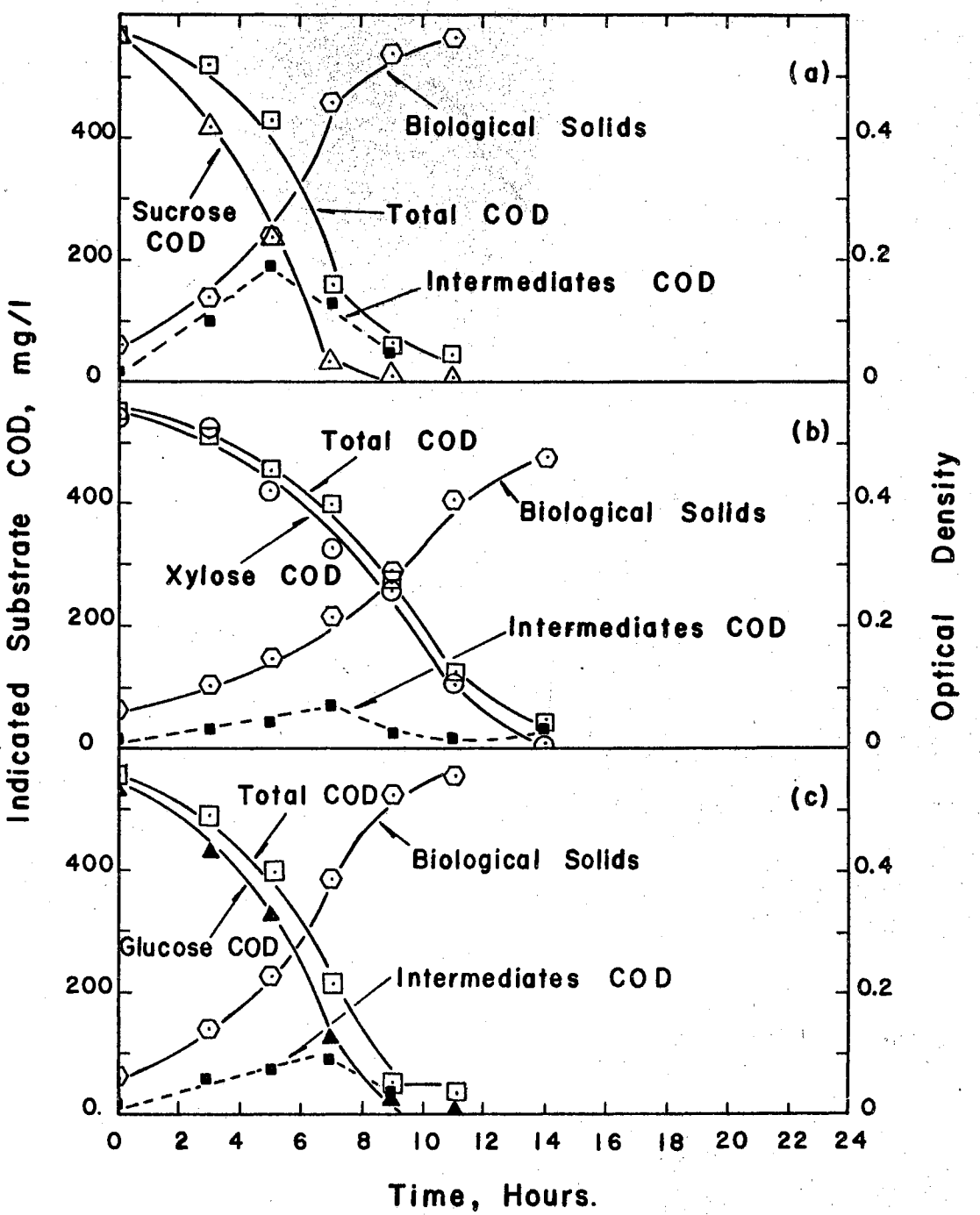

Figure 29 - System performance in the control units of (a) sucrose, (b) xylose, and (c) glucose; young cells acclimated to xylose.

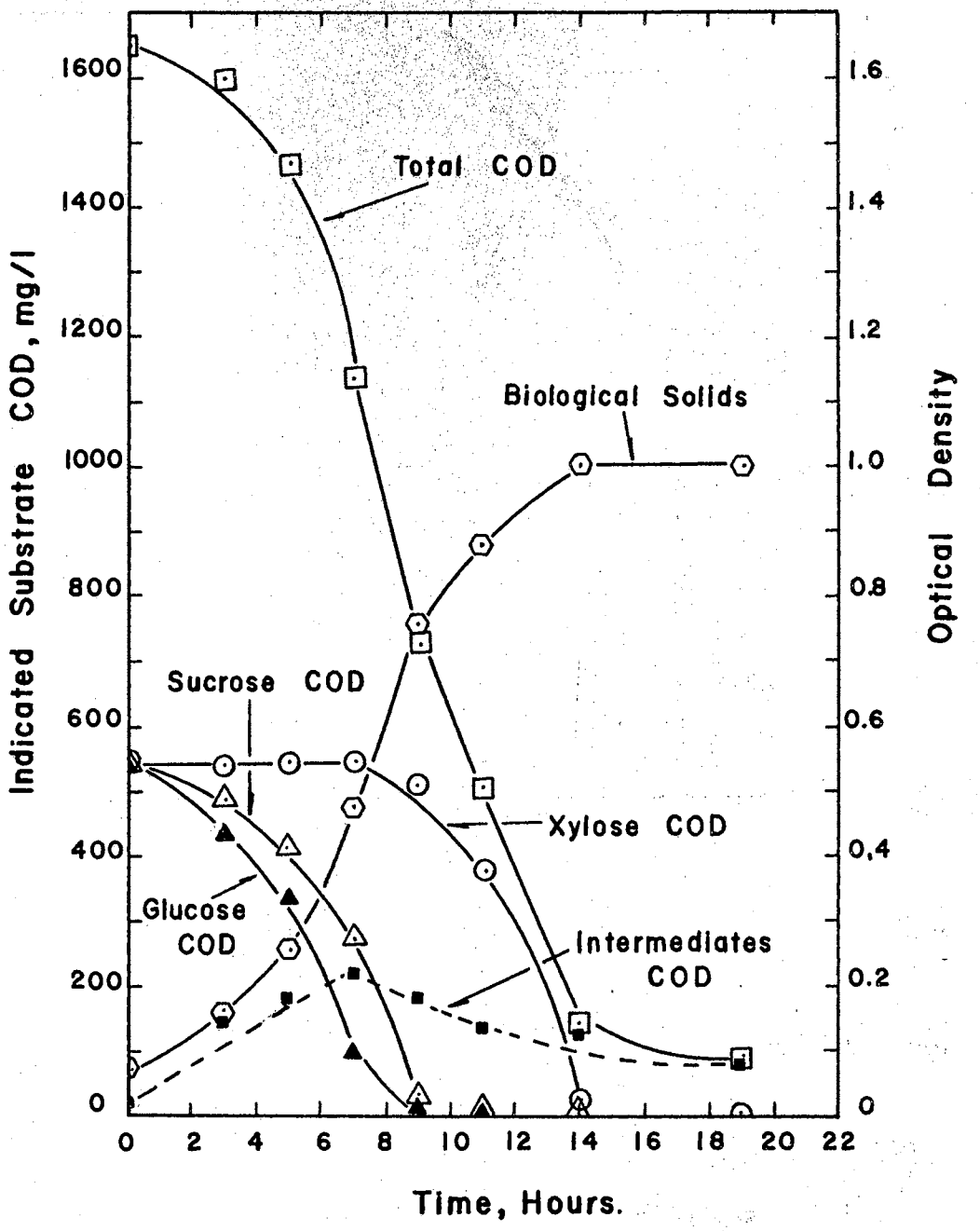

Figure 30 - System performance in the combined unit of glucose, xylose, and sucrose; young cells acclimated to xylose.

in the control (RT = 6.4 hrs) indicates that glucose metabolism was not affected by the presence of the other two sugars. Although sucrose possessed slightly higher potential for substrate interference than did glucose as adjudged from the results of the control units, sucrose removal was significantly retarded (from RT = 5.6 hrs in the control to 7.6 in the combined system) by the presence of glucose. It is noted that xylose catabolism did not start until more than 80 per cent of the glucose and 50 per cent of the sucrose had been consumed. The 7-hour lag for xylose metabolism suggests that the function of xylose-degrading enzymes was totally suppressed when glucose and sucrose were respectively greater than 20 per cent and 50 per cent that of xylose. Due to this interference, the operation time required for xylose purification was slightly increased (RT = 9.5 hrs in the control versus 12.9 hrs in the combined reactor).

c. Sucrose-acclimated Cells

The biological responses in the control units for cells grown on sucrose are presented in Figure 31. The rather long lag period for xylose metabolism indicates that the sucrose-grown cells did not contain the enzyme system for xylose catabolism at the initiation of the experiment. It is noted that the microbial population grew on glucose (RT = 10.4 hrs) and sucrose (RT = 10.6 hrs) more rapidly than on xylose (RT = 30.4 hrs). The logarithmic growth rate constants also supplied clear evidence of these growth responses. Based upon growth rate, accumulation of intermediates and acclimation requirement, the results for the control reactors indicate that the relative potential for interference is glucose > sucrose > xylose.

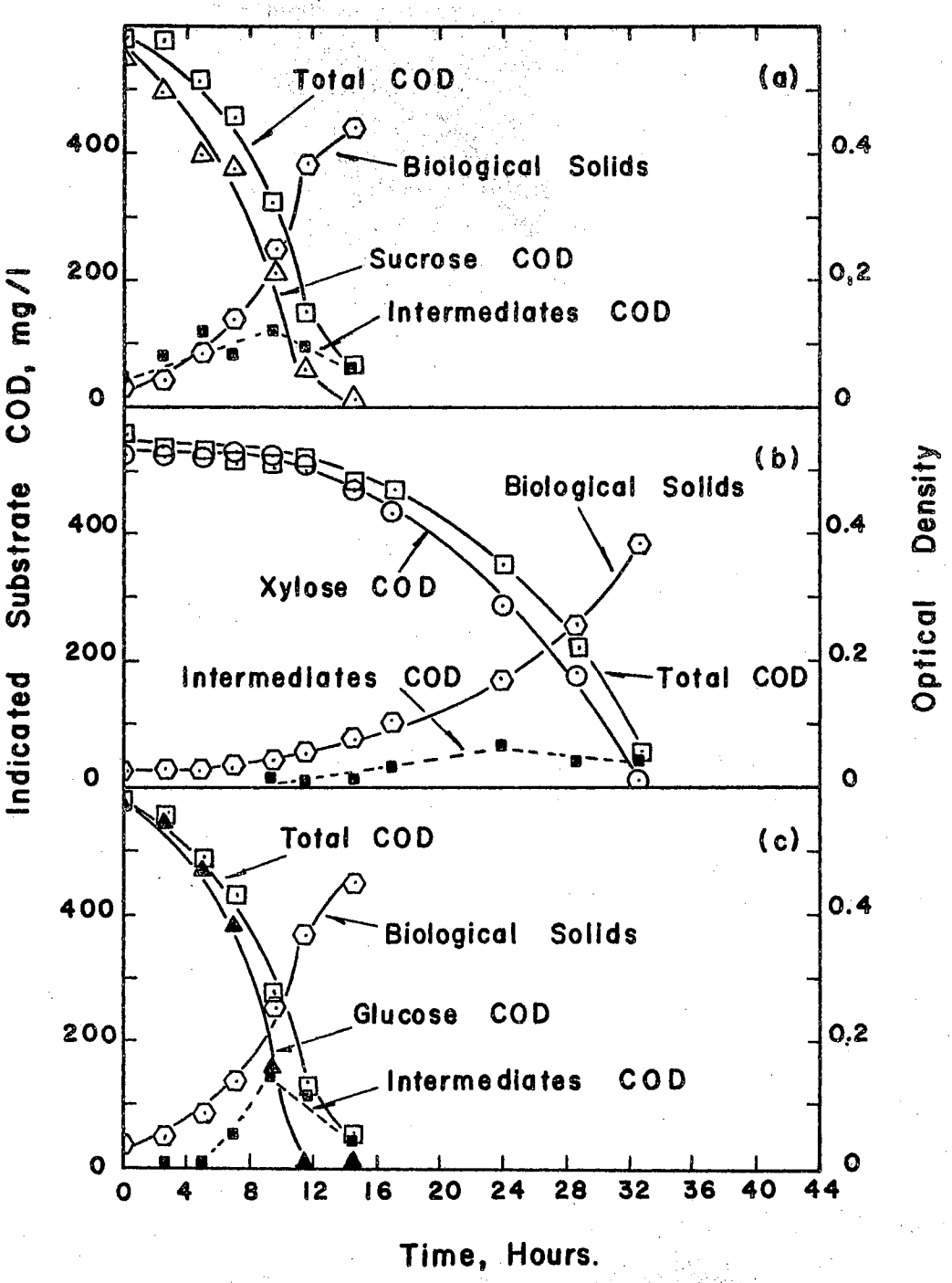

Figure 31 - System performance in the control units of (a) sucrose, (b) xylose, and (c) glucose; young cells acclimated to sucrose.

Figure 32 shows the responses in the mixed-substrate reactor. Comparison of glucose and sucrose removals in the combined reactor with those in the controls reveals that glucose metabolism was significantly enhanced (from RT = 9.2 to 7.2 hrs), in the presence of other sugars while sucrose utilization was slightly retarded (from RT = 9 to 10.4 hrs). This suggests that the functioning enzymes for sucrose catabolism might have been subject to a slight interference in the presence of glucose. The fact that the required lag period for xylose catabolism was not significantly changed in the combined reactor indicates that the synthesis of xylose-catabolizing enzymes was not retarded during the metabolism of the other two sugars. However, after the lag period, xylose metabolism in the combined system (RT = 16.8 hrs) was much more rapid than that in the control reactor (RT = 27.2 hrs). Since xylose removal started when glucose concentration was 220 mg/l (40 per cent of the xylose concentration) and since the peak accumulation of metabolic intermediates (about 520 mg/l) occurred when glucose was exhausted (9.5 hrs), it seems reasonable to conclude that the metabolic intermediates had little or no retarding effect on the catabolism of xylose.

As in the previous two experiments, the order of priority in metabolizing these three sugars was glucose > sucrose > xylose. Again, the sequence of removal was in accordance with the order of relative potential suggested by the results in the control reactors. From the results presented thus far, it appears that these three sugars were consumed in the order: glucose > sucrose > xylose, regardless of the past history of the cells, i.e., the process of acclimation did not change the sequence of utilization. This result was probably caused by a complete glucose inhibition of the xylose enzyme system when

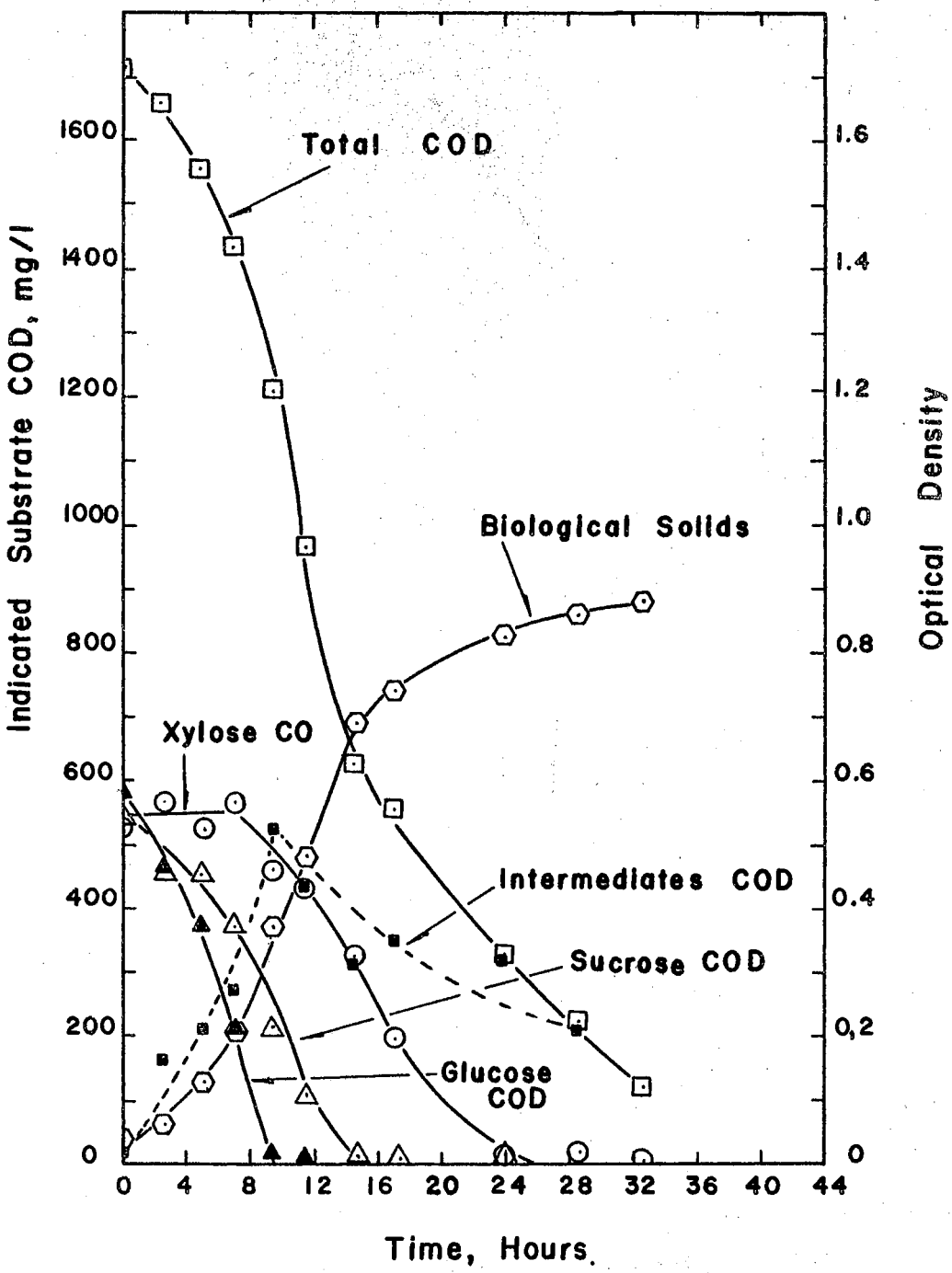

Figure 32 - System performance in the combined unit of glucose, xylose, and sucrose; young cells acclimated to sucrose.

glucose concentration was higher than a certain level, and a partial glucose inhibition of sucrose metabolism during the course of glucose catabolism.

II. Continuous Flow Experiments

The metabolic responses for the continuous flow system at dilution rates of 1/24, 1/12, 1/8, 1/6, 1/4, 1/3 and 1/2.5 hr^{-1} are shown in Figure 33. It is noted that this microbial population readily metabolized glucose, sucrose and xylose when the experiment was initiated at a 1/24 hr^{-1} dilution rate. The "steady-state" system efficiency at this dilution rate was 96 per cent. A high efficiency of substrate removal was ratained when the dilution rate was increased to 1/12 hr^{-1} (E=95 per cent) and to 1/8 hr^{-1} (E=97 per cent). During this period the cell yields were determined to be 42, 51.1 and 51 per cent, respectively, at dilution rates of 1/24, 1/12 and 1/8 hr^{-1}. The low cell yield at the dilution rate of 1/24 hr^{-1} might be attributable in part to incomplete-mixing of the reactor. However a more likely explanation is that at this low growth rate very little carbon would be channeled to storage products. Thus, the solids concentration level at this dilution rate was comparatively lower than those at 1/12 and 1/8 hr^{-1}.

A considerable disruption occurred when the flow rate was increased from 1/8 to 1/6 hr^{-1}. The biological solids dropped gradually after the increase in dilution rate and attained a new level (approximately 550 mg/l) in 6 days. During this period, the effluent COD fluctuated between 60 mg/l and 310 mg/l and then dropped to a fairly steady level of 200 mg/l. It was noted that the change in feeding rate also caused a change in predominating species as adjudged by a color change of the mixed liquor from light brown to dark brown. At times, a few cell

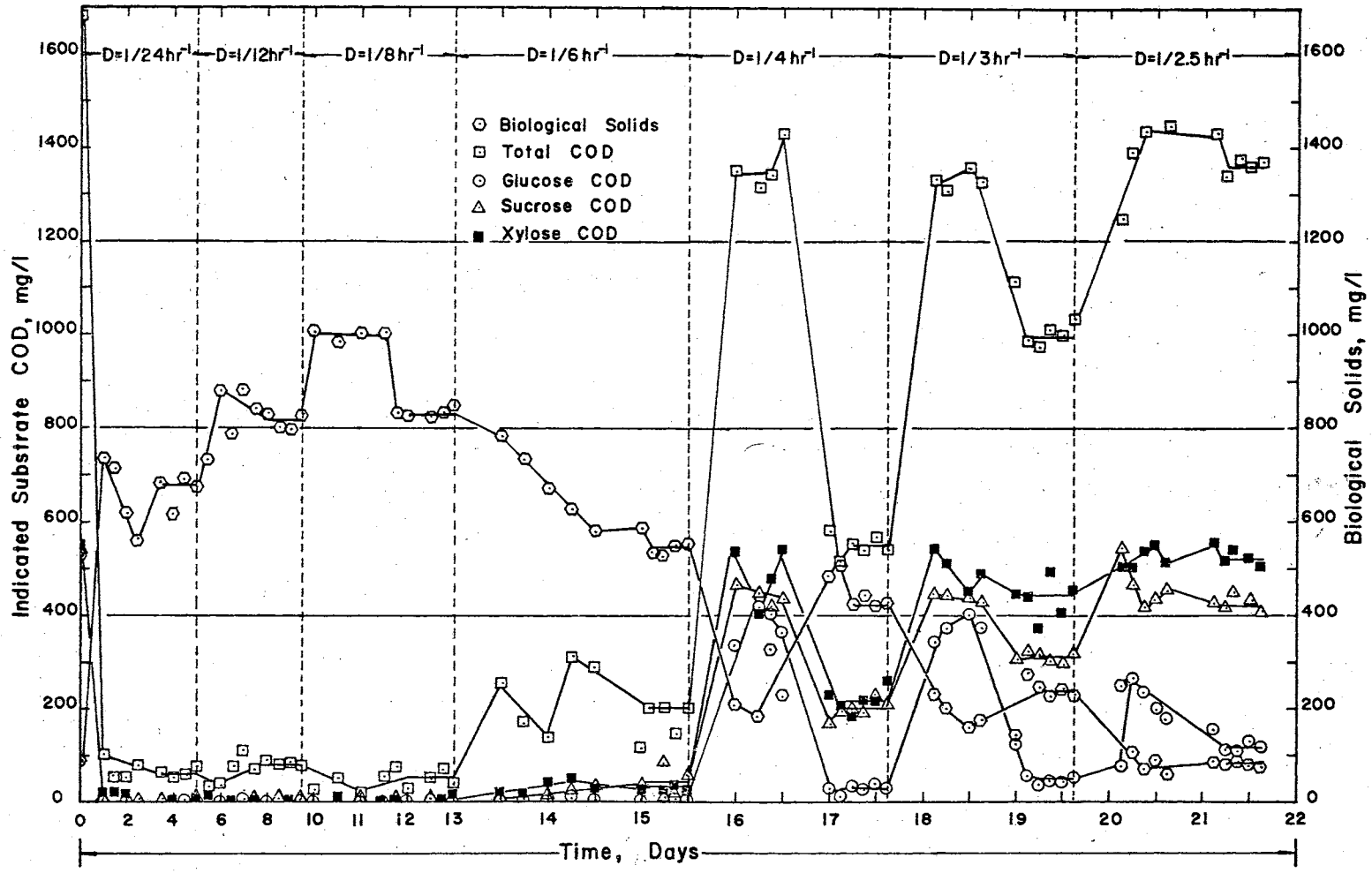

Figure 33 - System performance in the continuous flow activated sludge unit growing on glucose, sucrose, and xylose at various dilution rates.

aggregates were formed and prevented complete mixing in the reactor (for example, OD_r=1.011 vs. OD_e= 0.854 at 14 days and OD_r= 1.000 vs. OD_e = 0.831 at 15.5 days). Throughout operation at this flow rate, traces of xylose and sucrose (about 40 mg/l) were found in the effluent. The system efficiency observed at "steady-state" was 88 per cent.

A change in dilution rate from 1/6 to 1/4 hr^{-1} caused very severe transient disturbance to the system. The biological solids dropped to a minimum level of 180 mg/l and then built up to approximately 430 mg/l. The corresponding COD leakage attained a peak value of more than 1400 mg/l and then attained a new steady state level of approximately 550 mg/l within 2 days (12 detention periods). During the transient period, there was considerable fluctuation in the effluent concentrations of glucose, sucrose and xylose; the peak leakages were 410 mg/l, 470 mg/l and 540 mg/l respectively. However, they finally reached fairly constant levels of 30 mg/l, 200 mg/l and 220 mg/l, respectively. During this 2-day period of operation, the color of the mixed liquor gradually changed from dark brown to light greenish brown. It should be noted that the cells had a tendency to settle in the OD tube and it was impossible to adjudge the degree of complete mixing by measuring the optical densities of the reactor mixed liquor and the reactor effluent. At the near "steady-state" condition the cell yield value observed was 38 per cent, while the efficiency of substrate removal was 67 per cent, and it is apparent from the figure that both substrate and cells were being diluted out of the system to a greater extent than at the 1/6 hr^{-1} dilution rate.

When the flow rate was increased to 1/3 hr^{-1}, a severe transient disruption of system efficiency ensured. It was noted that the shock

load brought about gradual development of a few distinct small green flocs which may have militated against complete mixing of the reactor liquor. However the OD values in the reactor and in the effluent were 0.2903 and 0.2756 respectively at 19.5 days. Under "steady-state" conditions, the total COD leakage was approximately 1000 mg/l (450 mg/l xylose, 310 mg/l sucrose and 50 mg/l glucose and some metabolic intermediates). This significant leakage reduced the system efficiency to 60 per cent. The steady-state solids concentration was approximately 240 mg/l which provided a rather low cell yield of 35 per cent.

The last hydraulic shock load applied to this system was a change in dilution rate from 1/3 to 1/2.5 hr^{-1} which represented a 20 per cent increase in flow rate. It is seen that the system was severely disrupted by this shock load and exhibited little tendency to recover. Although the system was able to attain a fairly constant steady state within approximately 2 days (25 detention times), there appears to be little doubt that the system was operating close to the maximum growth rate. Thus this experiment was terminated upon attainment of that steady state. As for the foregoing dilution rate, the bulk of the stdady-state COD leakage (approximately 1360 mg/l) consisted of xylose (520 mg/l) and sucrose (420 mg/l); the remainder consisted of glucose (120 mg/l) and metabolic intermediates. The biological solids level at the termination of this experiment was about 80 mg/l. The cell yield was only 25 per cent and the treatment efficiency was 19 per cent.

The steady-state levels of the various parameters taken from Figure 33 for each dilution rate are given in Figure 34a. It is seen that all the carbon sources were completely consumed at dilution rates lower than 0.125 hr^{-1} (1/8 hr^{-1}). When the reactor was operating at

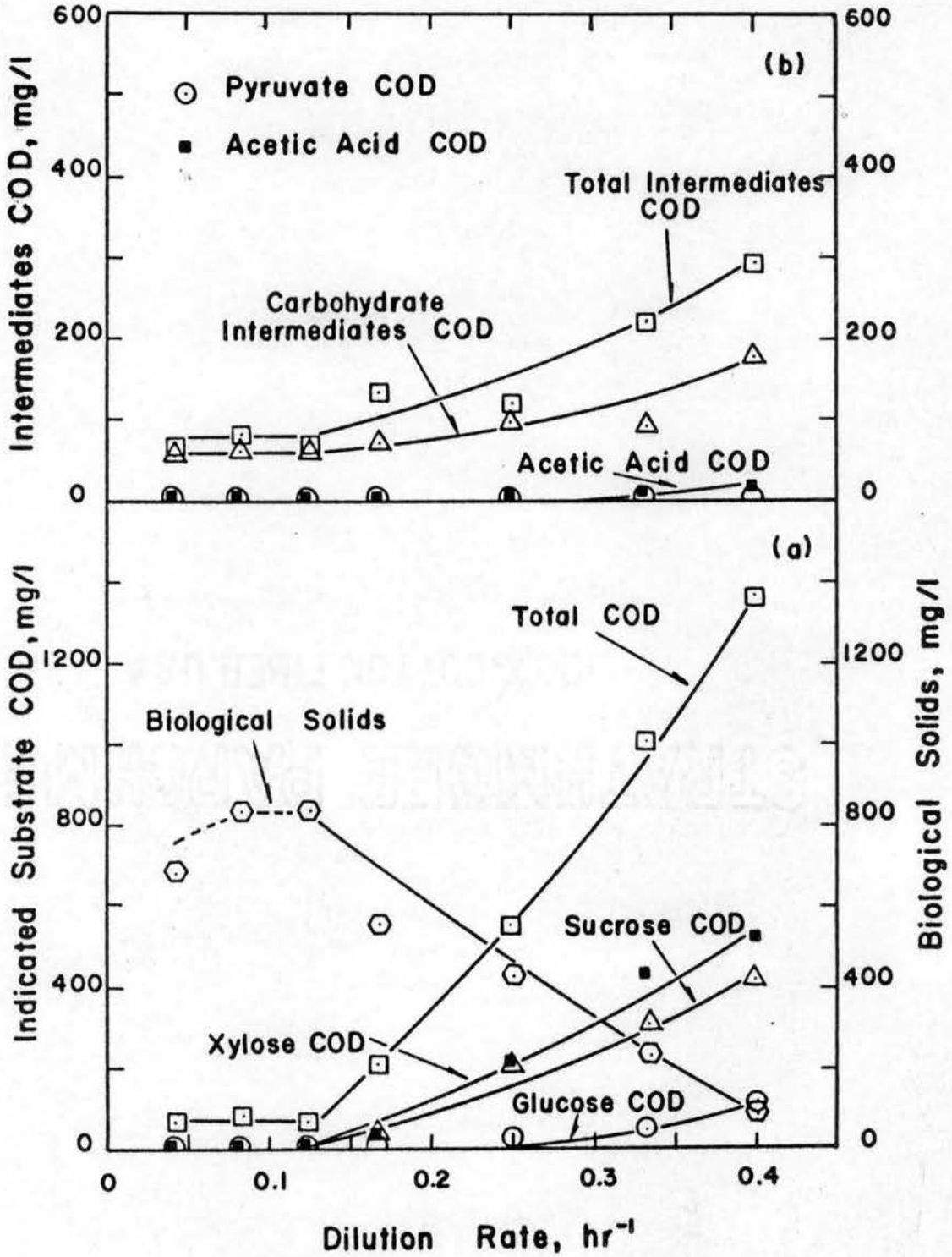

Figure 34 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, sucrose, and xylose at various dilution rates; (b) intermediates accumulation.

dilution rates exceeding 0.125 hr^{-1}, the system began to undergo dilute-out with respect to COD and solids. Xylose and sucrose began to appear in the effluent at the 1/6 hr^{-1} dilution rate. Glucose was the preferred carbon source in this system and was nearly completely consumed even at a dilution rate as high as 0.25 hr^{-1} (1/4 hr^{-1}). As the dilution rate was increased to 0.4 hr^{-1} (1/2.5 hr^{-1}), the efficiency of glucose removal was still rather high (79 per cent) but the efficiency of sucrose and xylose removal dropped to 21 per cent and 2 per cent respectively. It is interesting to note that the order of priority of substrate utilization was glucose > sucrose > xylose regardless of various changes in predominance which occurred during the experiment. The metabolic intermediate curves (Figure 34 b) show that the amount of accumulated intermediates was neglizible (80 mg/l) at low dilution rates, but the production of intermediates increased with increasing dilution rate. The total intermediate COD amounted to 290 mg/l at a dilution rate of 0.4 hr^{-1} and 59 per cent (170 mg/l) of these were accounted for as non-substrate carbohydrates. Only trace amounts of pyruvate (10 mg/l) and acetic acid (less than 10 mg/l) were found.

F. Studies on Substrate Removal in a Mixture of Glucose, Ribose and Glycerol by a Heterogeneous Population (Batch Studies Only)

a. Glucose-acclimated Cells

Results for the control units are shown in Figure 35. The patterns of biological growth shown in the figure as well as those of semilogarithmic plots of OD vs. time indicate that the cells acclimated rapidly to ribose and glycerol. However, the organisms grew on glucose (RT = 4.7 hrs) more rapidly than on glycerol (RT = 6.4 hrs) or ribose (RT =

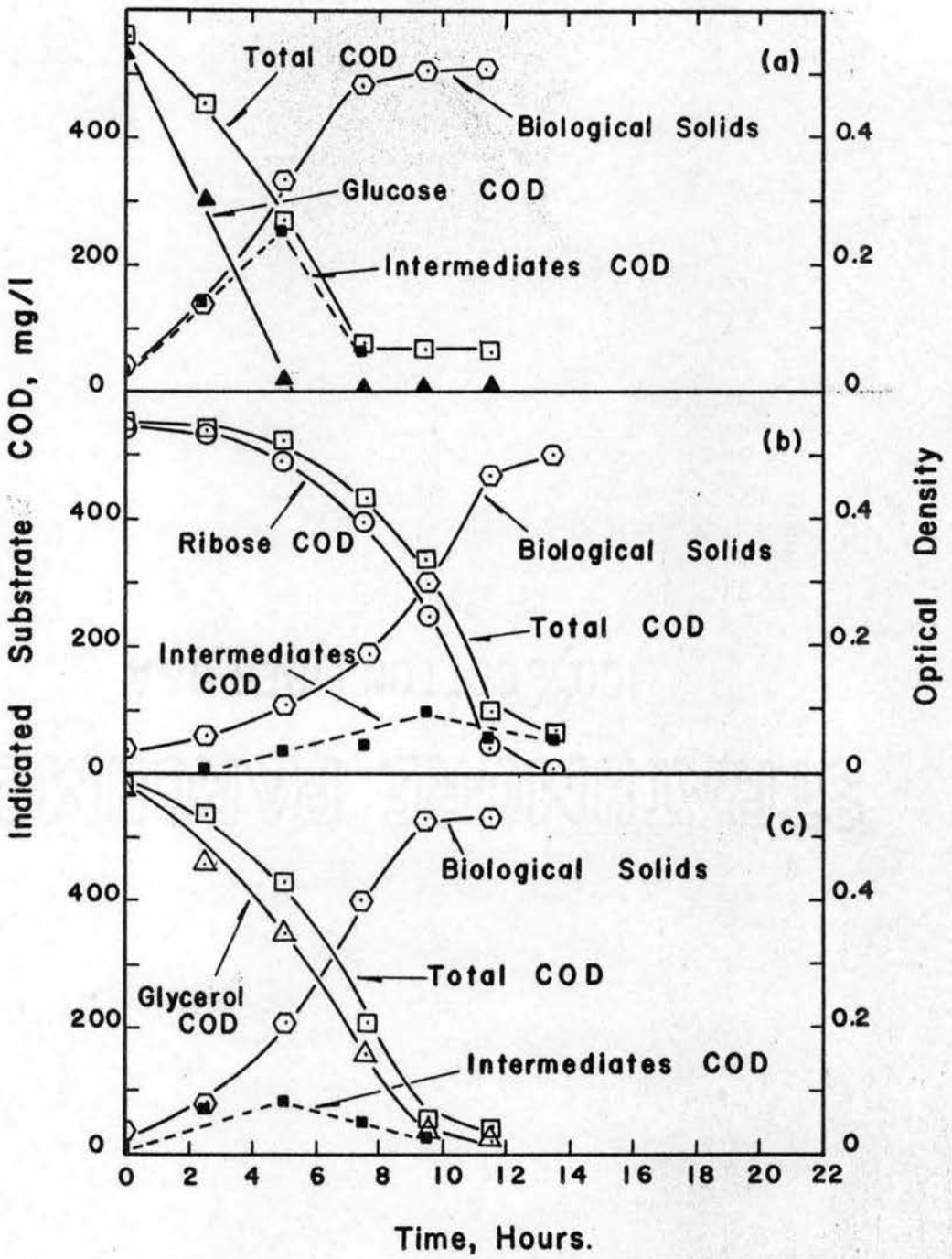

Figure 35 - System performance in the control units of (a) glucose, (b) ribose, and (c) glycerol; young cells acclimated to glucose.

9.4 hrs). The intermediate COD curves show that much more metabolic intermediates accumulated in the glucose reactor than in the other two units. Thus, based upon growth rate and intermediates accumulation, it is suggested that the potential ability for interference in a mixture of the three substrates would be glucose > glycerol > ribose.

When the three substrates were used as a combined carbon source (Figure 36), the sequence of substrate removal was in accordance with the potential ability for interference as suggested from the results of control units, i.e., glucose > glycerol > ribose. A comparison of glucose removal in the control (RT = 3.3 hrs) and the combined system (RT = 4.1 hrs) suggests that glucose metabolism was slightly retarded in the presence of other two substrates. The approximately equal "reference times" for biological growth in the glucose control and the combined system (RT =4.7 vs. 4.8 hrs) and the 5-hour blockage of ribose and glycerol removals indicate that glucose severely blocked the removal of both ribose and glycerol and that no significant removal of ribose or glycerol occurred during the metabolism of glucose. Since the cells may have contained enzyme systems for the metabolism of ribose and glycerol at the beginning of the experiment as adjudged from the results of control units (essentially no lag period), the blockages were most likely due to the inhibition of enzyme activity. Although glycerol and ribose were removed concurrently after release from the 5-hour glucose inhibition, glycerol was consumed (RT = 9.6 hrs) slightly faster than was ribose (RT = 10.6 hrs).

b. Ribose-acclimated Cells

The biological growth patterns for the control systems (Figure 37) as well as semi-logarithmic plots of OD vs. time indicate that the

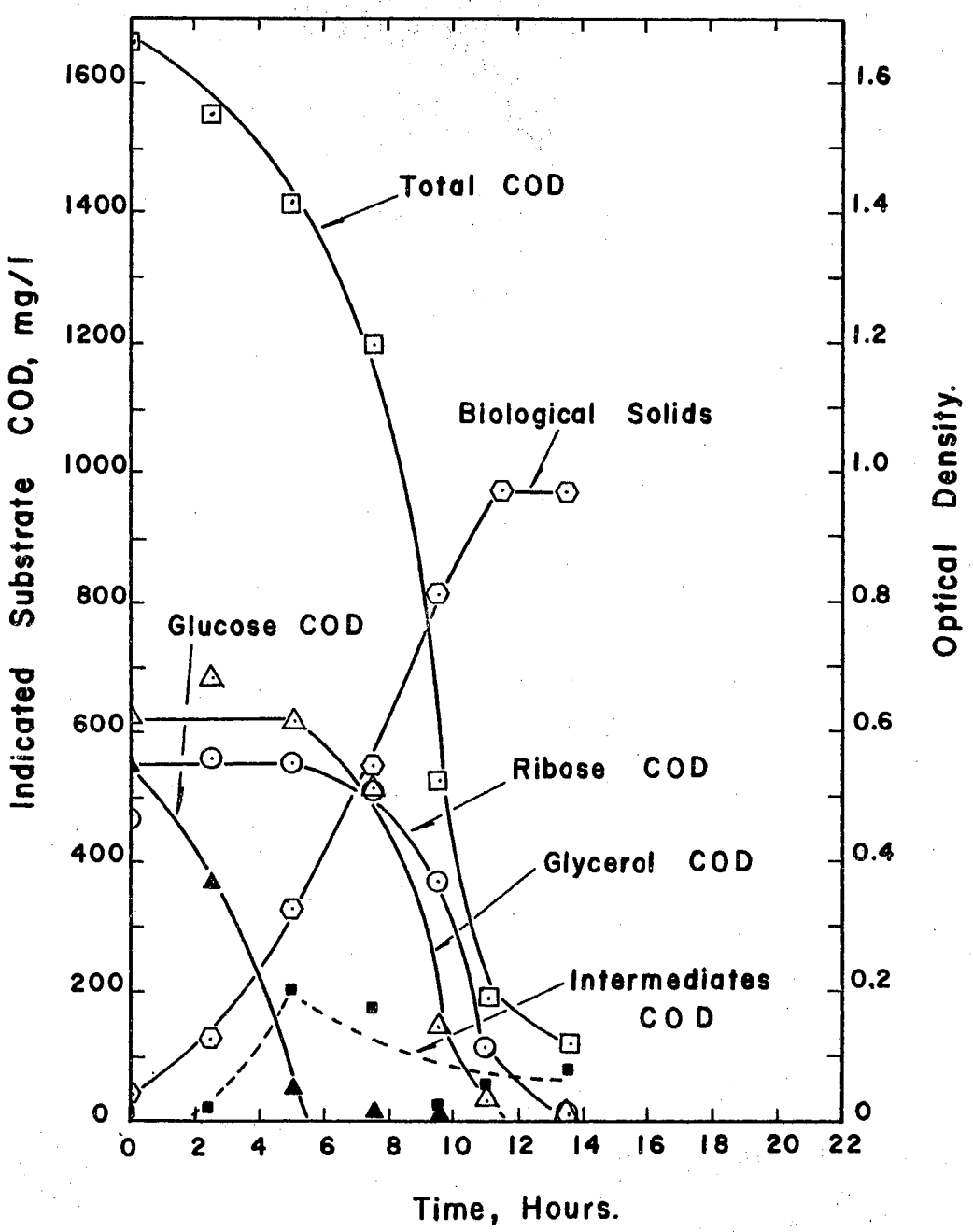

Figure 36 - System performance in the combined unit of glucose, ribose, and glycerol; young cells acclimated to glucose.

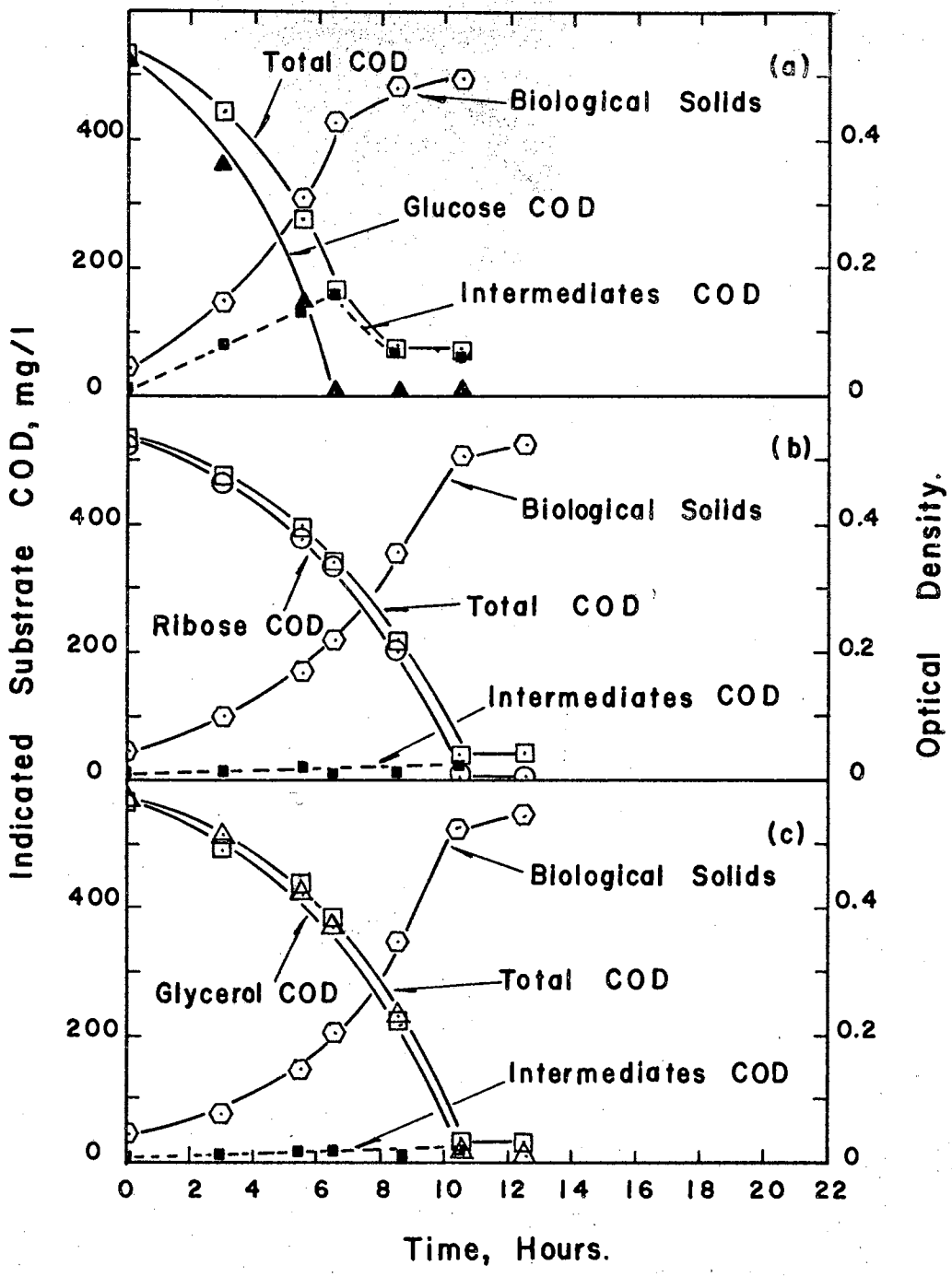

Figure 37 - System performance in the control units of (a) glucose, (b) ribose, and (c) glycerol; young cells acclimated to ribose.

ribose-grown cells acclimated rapidly or possessed constitutive enzymes for glucose and glycerol metabolism. Comparison of the rapidity of growth and the magnitude of intermediate accumulation in the glucose unit with the other two units suggests that glucose exhibits a rather high potential ability to interfere with the metabolism of the other two substrates when these three substrates make up the combined carbon source.

When the substrates were used as a combined carbon source (Figure 38), the removal of ribose and glycerol was very severely disrupted due to the presence of glucose. As with glucose-acclimated cells, these three substrates were eliminated in the order: glucose > glycerol > ribose. Glucose removal was only slightly retarded in the presence of other two substrates (glucose removal in the combined unit,RT = 5.6 hrs vs. that in the control, RT = 5.2 hrs). Ribose was released from glucose suppression earlier than was glycerol. Glycerol (RT = 9.6 hrs) was removed more rapidly than was ribose (RT = 10.6 hrs). This suggests that ribose metabolism was probably also subject to interference by glycerol after glucose was exhausted. Thus glycerol had greater potential for substrate interference than did ribose as had been observed in the previous experiments.

c. Glycerol-acclimated Cells

Results for the control units are shown in Figure 39. The biological growth curves shown in the figure as well as semi-logarithmic plots of OD vs. time show that the glycerol acclimated microbial population required little or no acclimation to glucose but required a considerable acclimation period on ribose. The cells grew on ribose (RT = 19.8 hrs) much more slowly than on glucose (RT = 8.2 hrs) or glycerol (RT =

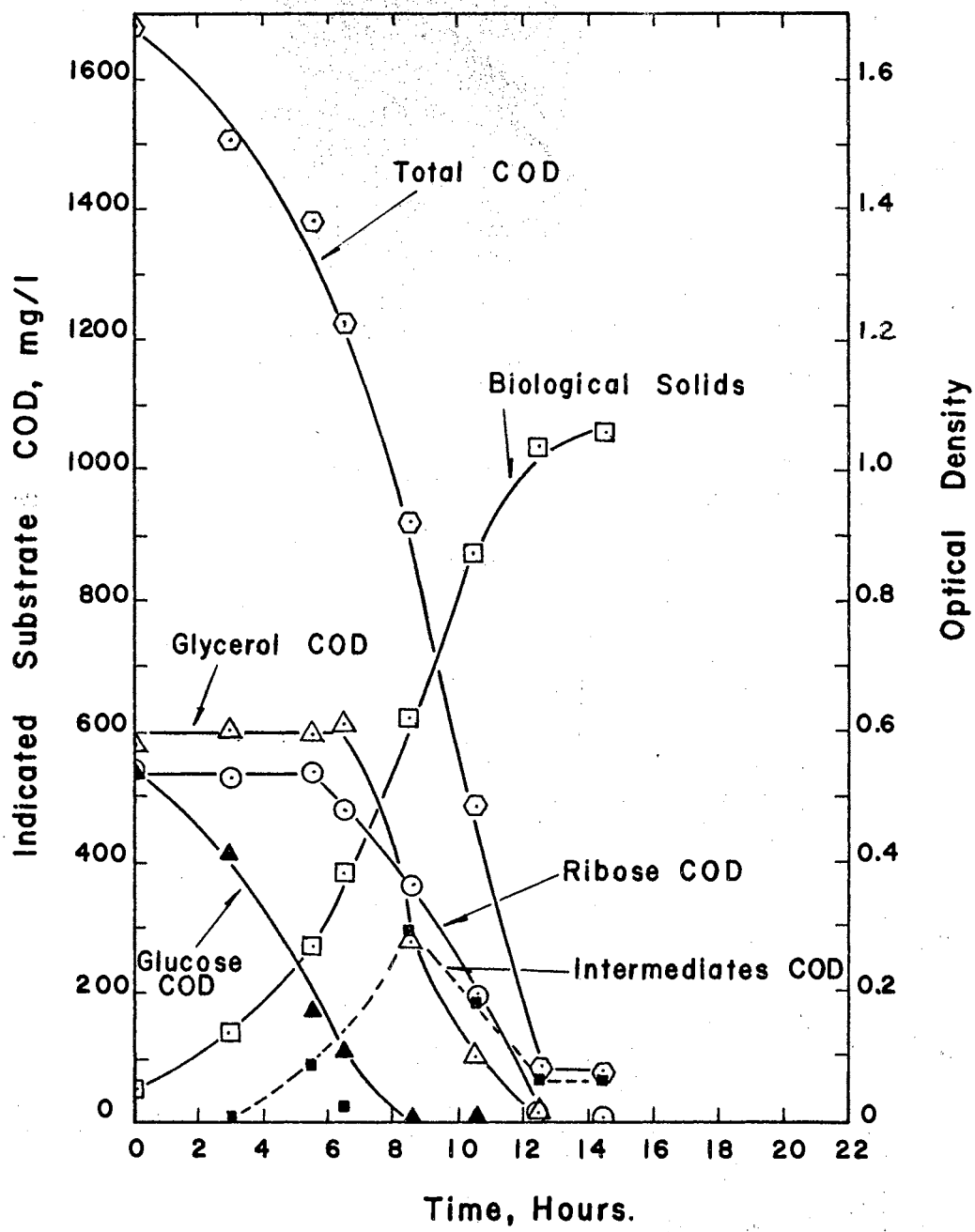

Figure 38 - System performance in the combined unit of glucose, ribose, and glycerol; young cells acclimated to ribose.

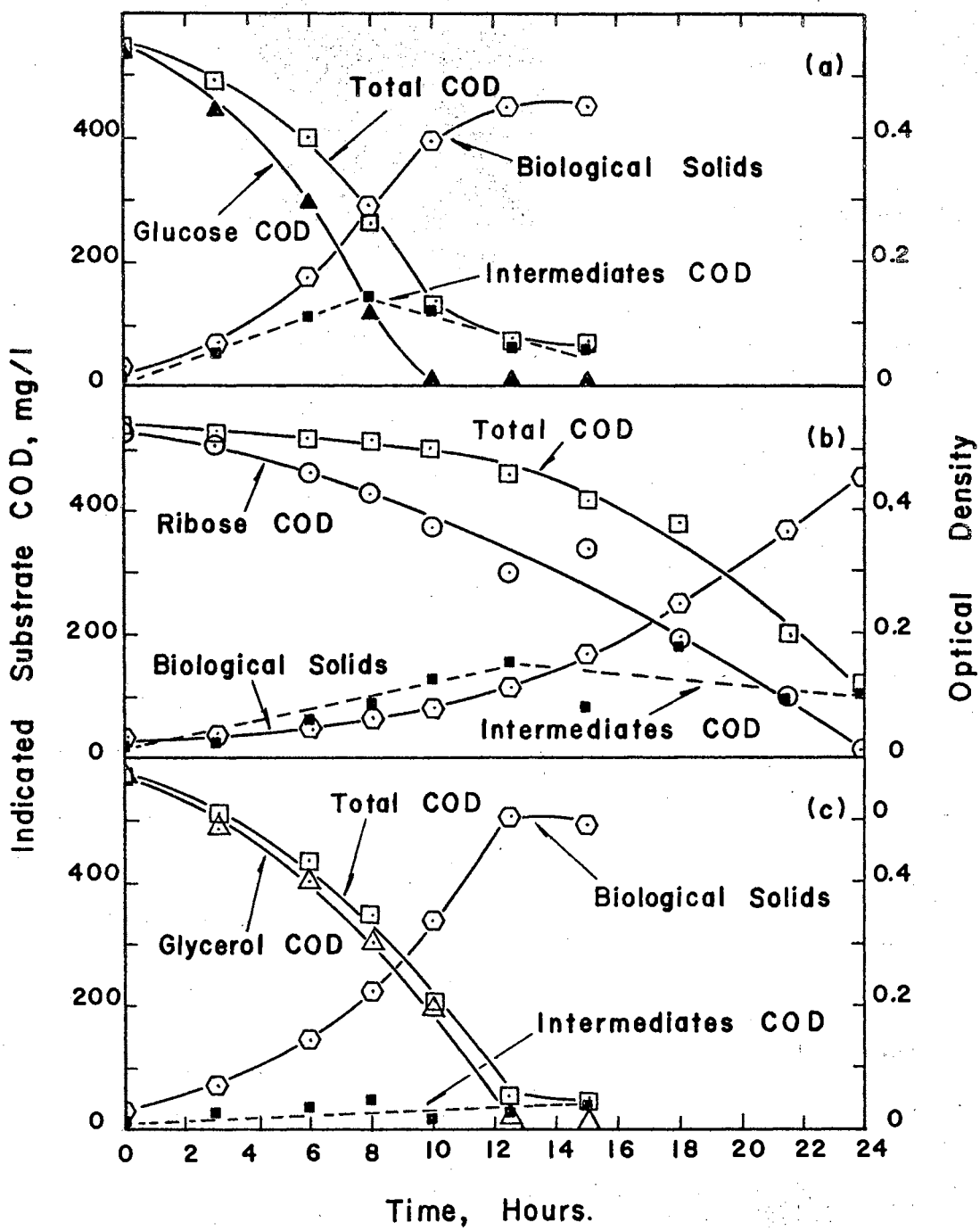

Figure 39 - System performance in the control units of (a) glucose, (b) ribose, and (c) glycerol; young cells acclimated to glycerol.

9.5 hrs) but they produced a somewhat greater amount of metabolic intermediates from ribose than from glucose and considerably more than from glycerol.

Figure 40 shows the metabolic responses in the combined system. As in the previous two experiments, glucose was consumed before the concurrent removal of ribose and glycerol started. However, the pattern of removal in this system (glucose > ribose > glycerol) was different from that shown in Figures 36 and 38 (glucose > glycerol > ribose). The general pattern of intermediate accumulation observed in the control reactors for ribose and glycerol and the rapid secondary buildup of intermediates after exhaustion of glucose in the combined system suggested that the buildup of intermediates was most likely due to ribose metabolism, and that these intermediates may have inhibited glycerol removal to a considerable extent (compare glycerol removal in Figure 38 with glycerol removal in Figure 40). Comparison of glucose removal and biological growth in the combined system (RT = 6.7 hrs and 7.2 hrs) with those in the glucose control system (RT = 7.2 hrs and 8.2 hrs) indicates that the cells in the combined reactor metabolized glucose slightly faster than did those in the control. The extended period of blockage of ribose and glycerol removal and the comparatively rapid removals of these two substrates after release from glucose suppression again support the contention that the removal of glycerol and ribose was severely inhibited by glucose, but the synthesis of the enzyme systems for utilizing these two substrates was apparently progressing during the metabolism of glucose. Due to the accumulation and retention of intermediates from ribose, the efficiency of COD removal in this system dropped to approximately 81 per cent (compared to 93 per cent and

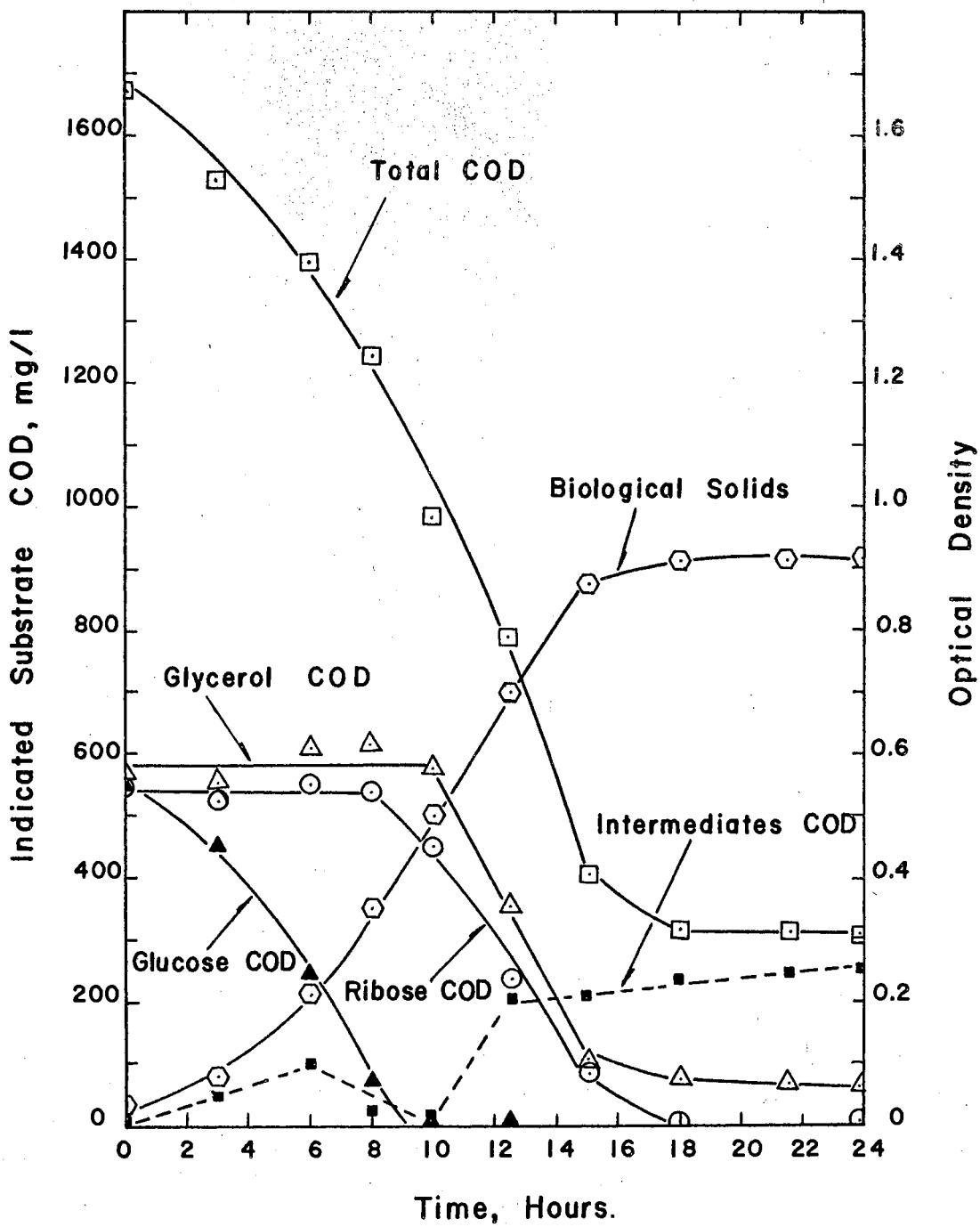

Figure 40 - System performance in the combined unit of glucose, ribose, and glycerol; young cells acclimated to glycerol.

95 per cent in Figures 36 and 38, respectively).

G. Studies on Substrate Removal in a Mixture of Glucose, Glycerol and Xylose by a Heterogeneous Population (Continuous Flow Reactor)

The performance of a system continuously cultured on a carbon source consisting of glucose-glycerol-xylose is shown in Figure 41. It is seen that glucose, xylose and glycerol were utilized readily by this population at a dilution rate of 1/12 hr^{-1}. Within 2 days of operation at this dilution rate, the system appeared to attain a steady-state condition. The optical density in the reactor was 1.046, and in the effluent 0.989; thus the reactor can be said to have been completely mixed. A fairly steady solids concentration of approximately 550 mg/l was attained and considerable leakage of COD, approximately 300 mg/l, was observed at this steady state. In the effluent only traces of xylose COD (approximately 30 mg/l) and glycerol COD (no more than 10 mg/l) were detected. The cell yield and system efficiency observed at steady-state were 40 per cent and 82 per cent, respectively.

After increasing the dilution rate to 1/8 hrs^{-1}, a significant system disruption occurred. There was considerable leakage in substrate during the well-defined transient state. The maximum substrate concentrations in the effluent during this period amounted to 230 mg/l xylose COD and 100 mg/l glycerol COD. After approximately 42 hours of operation at this dilution rate, the system regained a relatively steady-state; at this time the reactor was adjudged to be completely mixed (OD_e = 1.011 versus OD_r = 1.046). As in the transient state, the order of substrate leakage in the steady-state was xylose COD (90 mg/l) > glycerol COD (45 mg/l) > glucose (no glucose was detected). The steady-state cell yield and system efficiency were approximately 40 and

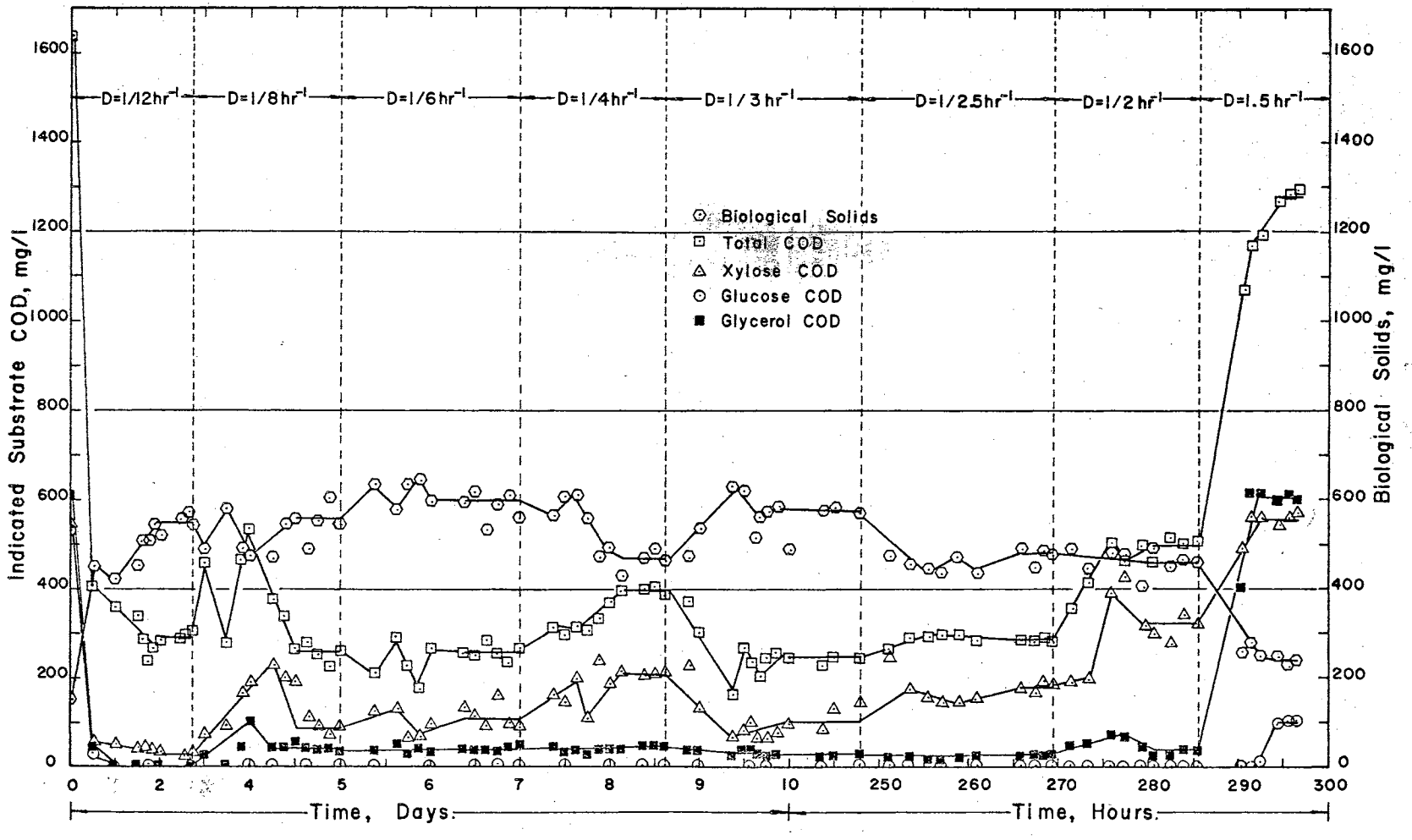

Figure 41 - System performance in the continuous flow activated sludge unit growing on glucose, glycerol, and xylose at various dilution rates.

84 per cent, respectively.

There were no significant changes in filtrate COD, substrate leakage or system efficiency when the dilution rate was changed to 1/6 hr^{-1}. The only change was the increase in cell yield to 43 per cent.

When the dilution rate was increased to 1/4 hr^{-1}, a gradual increase in COD leakage and a corresponding decrease in biological solids concentration were observed. It is noted that more than 28 hours (7 detention times) were required for the system to approach a new steady state. The approximate steady-state values were: 470 mg/l biological solids, 400 mg/l effluent COD, 200 mg/l xylose COD, and 50 mg/l glycerol COD. The cell yield and system efficiency were depressed to approximately 37 and 76, respectively.

The dilution rate was next increased to 1/3 hr^{-1}. It is interesting to note that the increase in dilution rate brought about an increase in biological solids concentration (from a steady-state value of 470 mg/l to 580 mg/l) and a better utilization of the carbon source (from a steady-state COD value of 400 mg/l to 250 mg/l). It was also noted that the appearance of the sludge changed from a population which was dispersed and imparted a grayish color to the mixed liquor to one which was somewhat flocculated and imparted a greenish color to the aeration liquor. The increases in solids concentration and COD utilization were probably due, to a small extent, to solids retention in the reactor. At 245 hours, the OD in the reactor was 1.034 compared to 0.949 in the effluent. During "steady-state" the approximate substrate leakages were : 100 mg/l xylose COD and 30 mg/l glycerol COD. Corresponding to the changes in solids concentration and effluent COD, the cell yield and system efficiency again rose to 41 and 85 per cent, respectively.

A slight increase in COD leakage and a slight decrease in biological solids concentration occurred when the dilution rate was changed from 1/3 hr^{-1} to 1/2.5 hr^{-1}. Approximately 18 hours (more than 7 detention times) were required to attain a new steady-state. At the new "steady-state," glucose was still not detectable in the effluent and glycerol COD was maintained at the same level (30 mg/l) as at the previous dilution rate; however, xylose COD rose to approximately 180 mg/l. The steady-state cell yield at this dilution rate was determined to be 35 per cent, while 83 per cent of the COD was removed. At the end of operation at this dilution rate (269.5 hrs), the optical densities were OD_e = 0.894 versus OD_r = 0.803; thus the reactor cannot be said to have been completely mixed.

When the dilution rate was changed to 1/2 hr^{-1}, there was considerable increase in COD leakage during the transient state, but no significant change in solids concentration was observed throughout operation at this flow rate. During the transient state period, the maximum substrate concentration in the effluent amounted to 390 mg/l xylose COD and 70 mg/l glycerol COD. About 10 hours (5 detention times) were required for the system to attain a new steady behavior. At this time the unit showed a fairly good degree of complete mixing (e.g., OD_r = 0.776 versus OD_e = 0.745 at 280.5 hrs).

When the dilution rate was increased to 1/1.5 hr^{-1}, the carbon source was rapidly "washed-out;" the leakage was approximately 1280 mg/l COD. The biological solids were "diluted-out" to approximately 24 mg/l. Also it appeared that the hydraulic shock brought about a change of the predominating species in the reactor, as evidenced by the development of greenish, flocculated, filamentous organisms. During

this time the reactor exhibited a certain degree of incomplete mixing as adjudged by the retention of a few flocs in the reactor and the higher optical density in the reactor than in the effluent (e.g., OD_r = 585 versus OD_e = 0.516 at the end of the experiment). It should be noted that samples during operation at this dilution rate were taken directly from the reactor. About 9 hours (6 detention times) after the change in dilution rate, the system approached an approximate steady-state condition with respect to biological solids concentration and effluent COD. Only 23 per cent of the total COD was removed, but the cell yield was increased to 61 per cent. While this was the highest value for cell yield at any of the dilution rates, the fact that the system was not completely mixed detracts from the credibility of this value.

Figure 42 shows the average steady-state parameters and production of intermediates at the various dilution rates. Glucose was the preferred carbon source and was completely utilized even at a dilution rate as high as 1/2 hr^{-1}. At a dilution rate of 1/1.5 hr^{-1}, only about 19 per cent of the glucose remained unutilized. Glycerol was also used efficiently at high dilution rates. Although traces of glycerol COD (less than 50 mg/l) were detected at dilution rates lower than 0.5 hr^{-1}, these glycerol COD's may have been contributed by periodate-reactive intermediates rather than the original glycerol in the feed. Except for the complete dilute-out at the dilution rate of 1/1.5 hr^{-1} (0.667 hr^{-1}), most of the glycerol was utilized in this experiment. There is no doubt that xylose was the least utilized substrate in this system. The leakage of xylose COD started at the lowest dilution rate (1/12 hr^{-1}), and gradually increased with increases in dilution rate. It is interesting

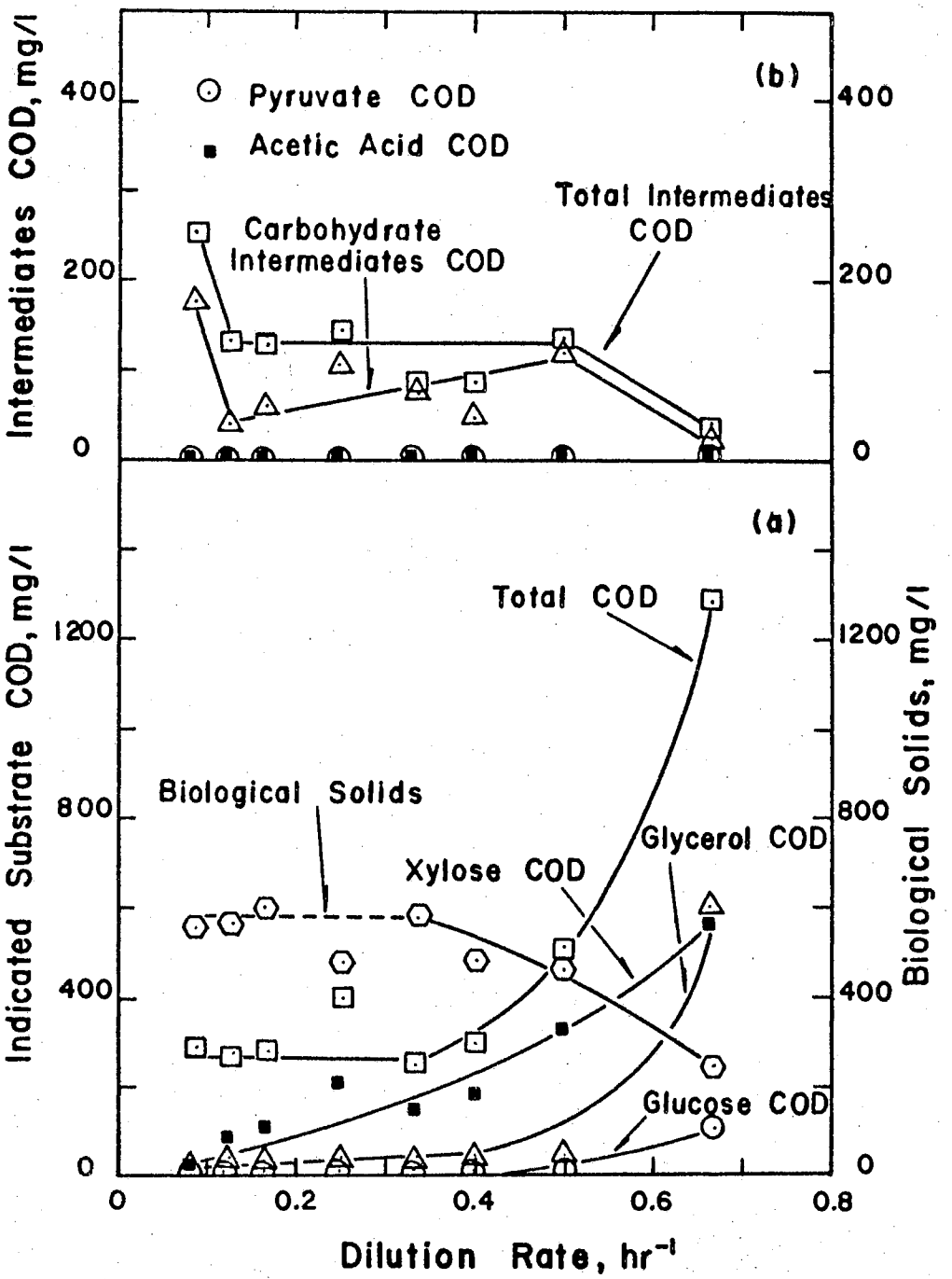

Figure 42 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, glycerol, and xylose at various dilution rates; (b) intermediates accumulation.

to note that the levels of solids concentration and effluent COD were somewhat constant for dilution rates lower than 1/3 hr^{-1} (0.333 hr^{-1}), regardless of increasing xylose leakage. These data indicate that the order of substrate removal was glucose > glycerol > xylose, regardless of various changes in predominance.

Figure 42b shows that the production of metabolic intermediates fluctuated throughout the experiment. Surprisingly, the maximum excretion of intermediates (250 mg/l) occurred at the lowest dilution rate (1/12 hr^{-1}), while the minimum (20 mg/l) occurred at the highest dilution rate (1/1.5 hr^{-1}).

H. Studies on Substrate Removal in a Mixture of Glucose, Sucrose and Glycerol by a Heterogeneous Population (Batch Studies Only)

a. Glucose-acclimated Cells

The course of growth and substrate removal for the control units are shown in Figure 43. During preparation of the stock solutions, a miscalculation was made in the amounts of sucrose and glycerol required, which accounts for the low initial COD values for these two substrates in the control (and in the combined systems as well, Figure 44). Figure 43 shows that no difficulty was encountered in utilizing sucrose and glycerol. However, growth on glucose (RT = 5.8 hrs) and sucrose (RT = 6.2 hrs) was significantly faster than was growth on glycerol (RT = 10.5 hrs). It also can be seen that slightly more intermediates accumulated during glucose and sucrose metabolism than during growth on glycerol. These observations suggest that the relative capability for substrate interference in a mixture of these three substrates would be glucose > sucrose > glycerol.

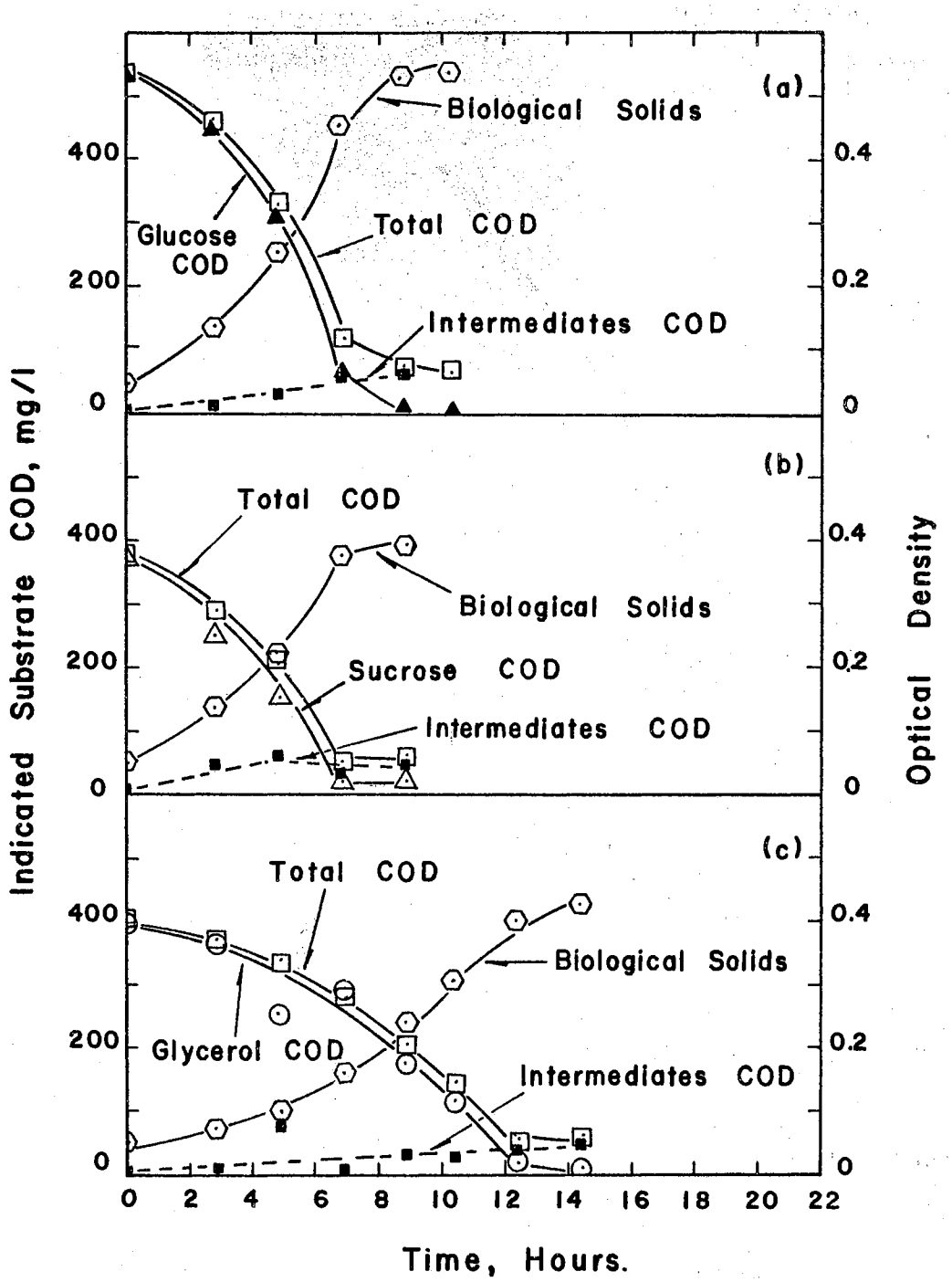

Figure 43 - System performance in the control units of (a) glucose, (b) sucrose, and (c) glycerol; young cells acclimated to glucose.

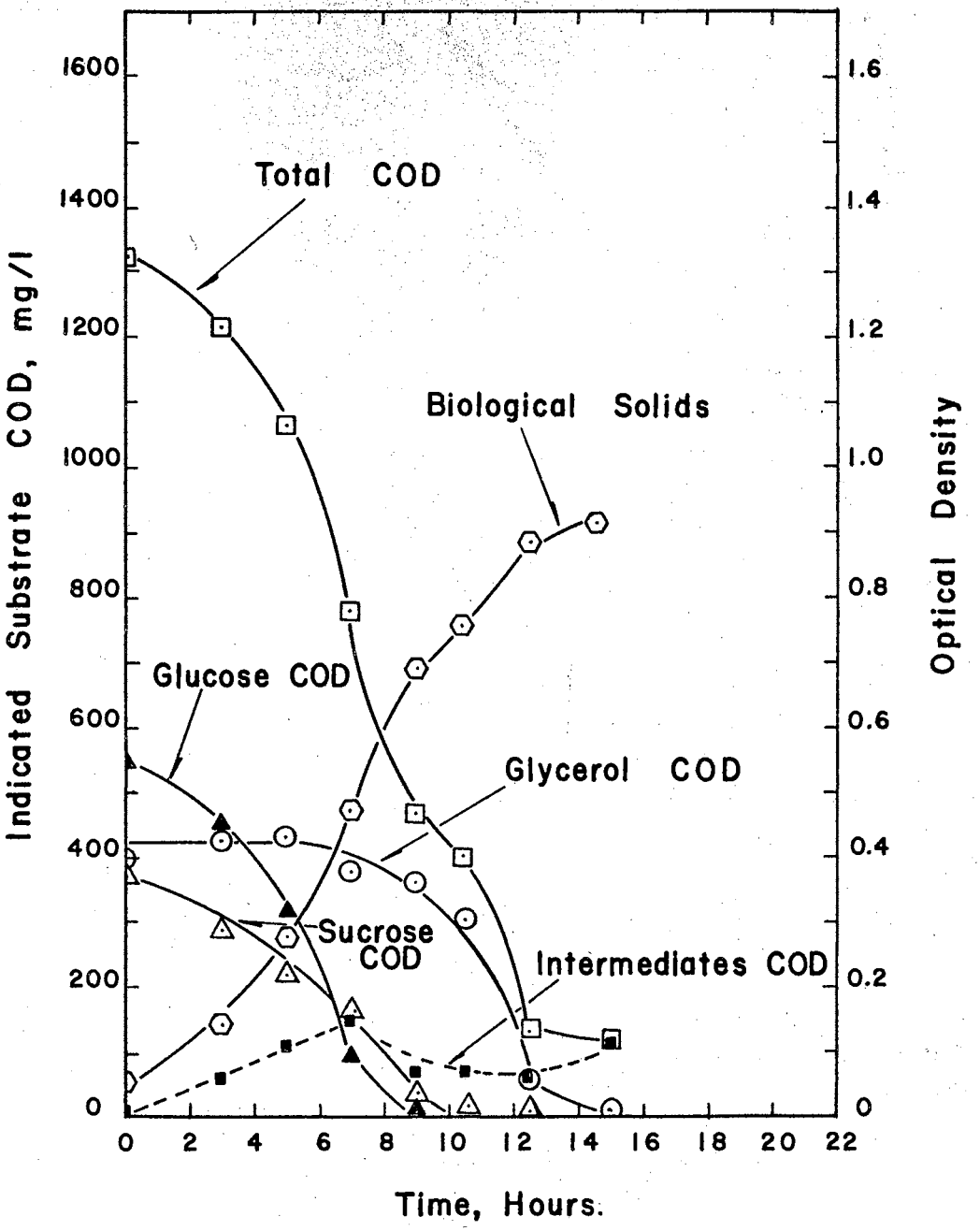

Figure 44 - System performance in the combined unit of glucose, sucrose, and glycerol; young cells acclimated to glucose.

When these three substrates were used as combined carbon source (Figure 44), the order of priority of utilization was glucose > sucrose > glycerol. Semilogarithmic plots of total COD removal versus time revealed that the total COD consumption and biological growth were distinctly diphasic in character. The first phase was associated primarily with concurrent removal of glucose and sucrose, whereas the second phase corresponded to removal of glycerol. A comparison of glucose and sucrose removals in the combined (RT_g = 6.3 hrs, RT_s = 6.0 hrs) with those in the controls (RT_g = 6.0 hrs, RT_s = 4.3 hrs) indicates that glucose metabolism was only slightly influenced by the other two substrates, but sucrose removal was significantly retarded in the combined unit. The pronounced lag period (more than 5 hours) for glycerol removal was a clear indication that glycerol metabolism was totally blocked by the presence of glucose and sucrose. Essentially no glycerol had been consumed by the time about half of the glucose and sucrose were removed from the reactor. When most of the glucose and sucrose had been used (at 9 hrs) approximately 83 per cent of the glycerol still remained unutilized.

b. Sucrose-acclimated Cells

Growth and substrate removal patterns in the control systems are shown in Figure 45. No significant acclimation period was required for the cells to use glucose or glycerol, and approximately 100 mg/l of intermediates were accumulated in each of the three reactors. A comparison of growth on these three substrates (RT_g = 5.6, RT_s = 6.5, and RT_{gly} = 9.7 hrs) suggests that the relative capability for substrate interference would be similar to that found for glucose-acclimated cells (Figure 43), i.e., glucose > sucrose > glycerol.

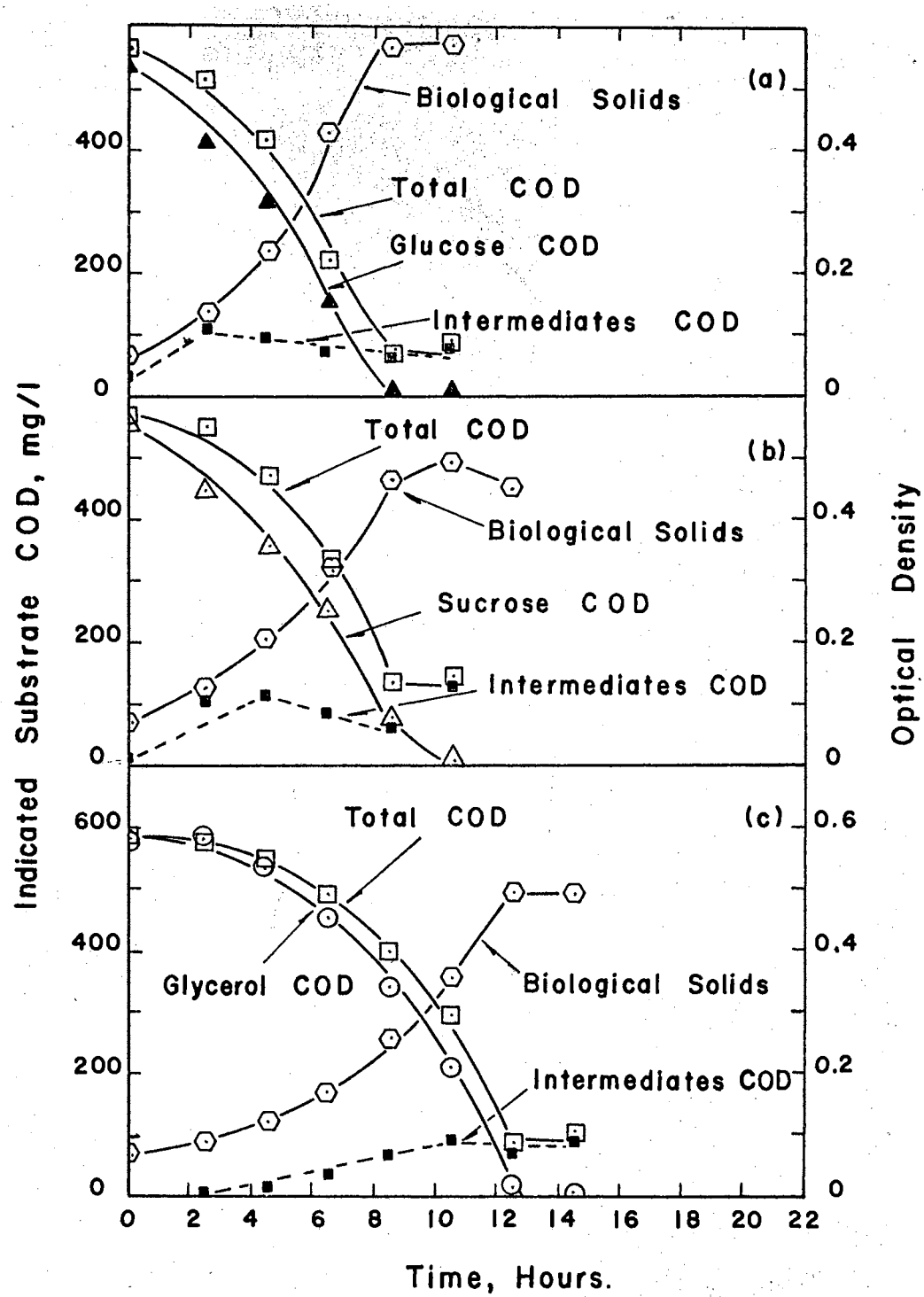

Figure 45 - System performance in the control units of (a) glucose, (b) sucrose, and (c) glycerol; young cells acclimated to sucrose.

The order of priority for substrate removal in the combined system (Figure 46) was glucose > sucrose > glycerol, which was in accordance with the relative capability sequence observed from the control results; the results are in general similar to those of the previous experiment (Figure 44). It is noted that both total COD removal and biological growth exhibited a distinctly diphasic character. Apparently the first phase was due primarily to the concurrent removal of glucose and sucrose, whereas the subsequent glycerol removal caused the second-phase metabolism. Glucose was metabolized somewhat more slowly in the combined reactor (RT = 7.1 hrs) as compared to the control (RT = 6.1 hrs), while removal of sucrose was greatly retarded in the presence of the other two substrates (from RT = 7.0 hrs to RT = 8.6 hrs). From Figure 46 it can be seen that while glucose retarded sucrose removal, it did not stop it, indicating that the activity of the preformed enzymes (cells were acclimated to sucrose) was severely blocked by glucose metabolism. The inhibition of sucrose removal was released when approximately half of the glucose had been exhausted, thereafter sucrose was rapidly utilized (K_s = 115 mg/l hr, calculated as a zero order rate). The fact that glycerol removal did not start until both sucrose and the accumulated intermediates, which were produced after glucose exhaustion, had been consumed indicates that glycerol removal was probably also subject to the influence of sucrose and/or the accumulated intermediates produced from sucrose.

c. Glycerol-acclimated Cells

In the control systems (Figure 47), the cells grew on glucose (RT = 5.5 hrs) only slightly faster than on glycerol (RT = 6.0 hrs) and sucrose (RT = 6.3 hrs). Since more intermediates were accumulated in

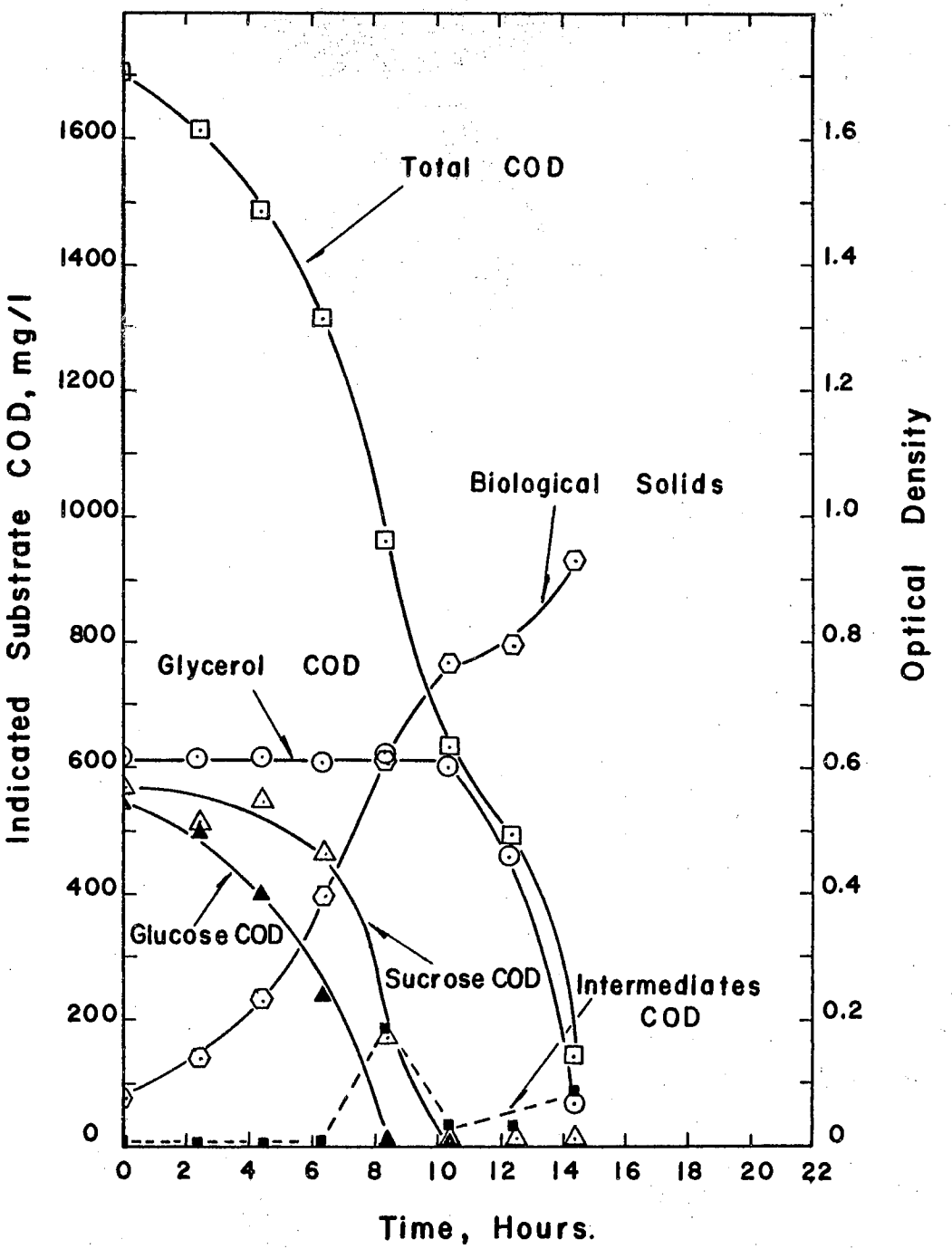

Figure 46 - System performance in the combined unit of glucose, sucrose, and glycerol; young cells acclimated to sucrose.

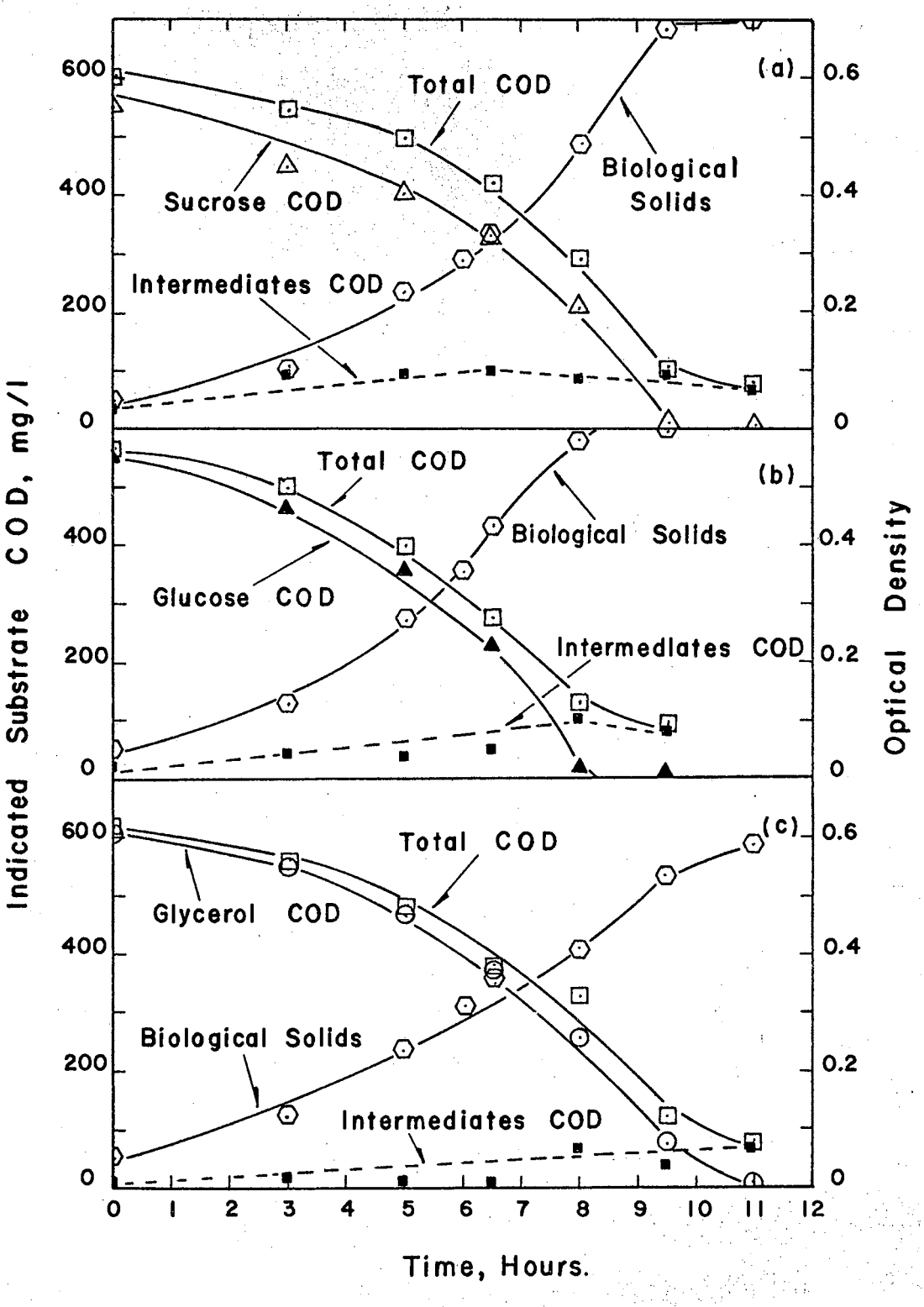

Figure 47 - System performance in the control units of (a) sucrose, (b) glucose, and (c) glycerol; young cells acclimated to glycerol.

the sucrose reactor than from glycerol metabolism, sucrose might be expected to exert an effect on glycerol metabolism.

Then the compounds were used as a combined carbon source (Figure 48), the removal of both sucrose and glycerol was very severely blocked by the presence of glucose; after the exhaustion of glucose, concurrent metabolism of sucrose and glycerol proceeded rather rapidly. Comparing biological growth and glucose removal in the combined system (RT_b = 4.6 hrs and RT_g = 6.0 hrs) with the glucose control (RT_b = 5.5 hrs and RT_g = 6.7 hrs) indicates that glucose removal was slightly retarded in the combined system, but small amounts of glycerol and sucrose were used and contributed to growth during glucose metabolism. It is seen that when glucose was exhausted, approximately 70 mg/l of sucrose and 150 mg/l of glycerol had been consumed. Although sucrose was released from glucose blockage 1.5 hrs later than was glycerol, sucrose was catabolized more rapidly (RT = 9.6 hrs) than was glycerol (RT = 10.7 hrs). Thus glycerol removal was also subject to sucrose interference, but glycerol had no apparent effect on sucrose metabolism. As in the previous two experiments, the sequence of substrate consumption was glucose > sucrose > glycerol. However, the pattern of sequential removal in this system (glucose consumption, then concurrent removal of sucrose and glycerol) was quite different from that shown in Figures 44 and 46 (concurrent removal of glucose and sucrose, then glycerol consumption).

I. Studies on Substrate Removal in a Mixture of Glucose, Sorbitol and Propionic Acid (Continuous Flow Reactor)

The metabolic responses for this substrate system under continuous operation at various dilution rates are presented in Figure 49. Upon addition of the acclimated cells, the substrates were rapidly utilized

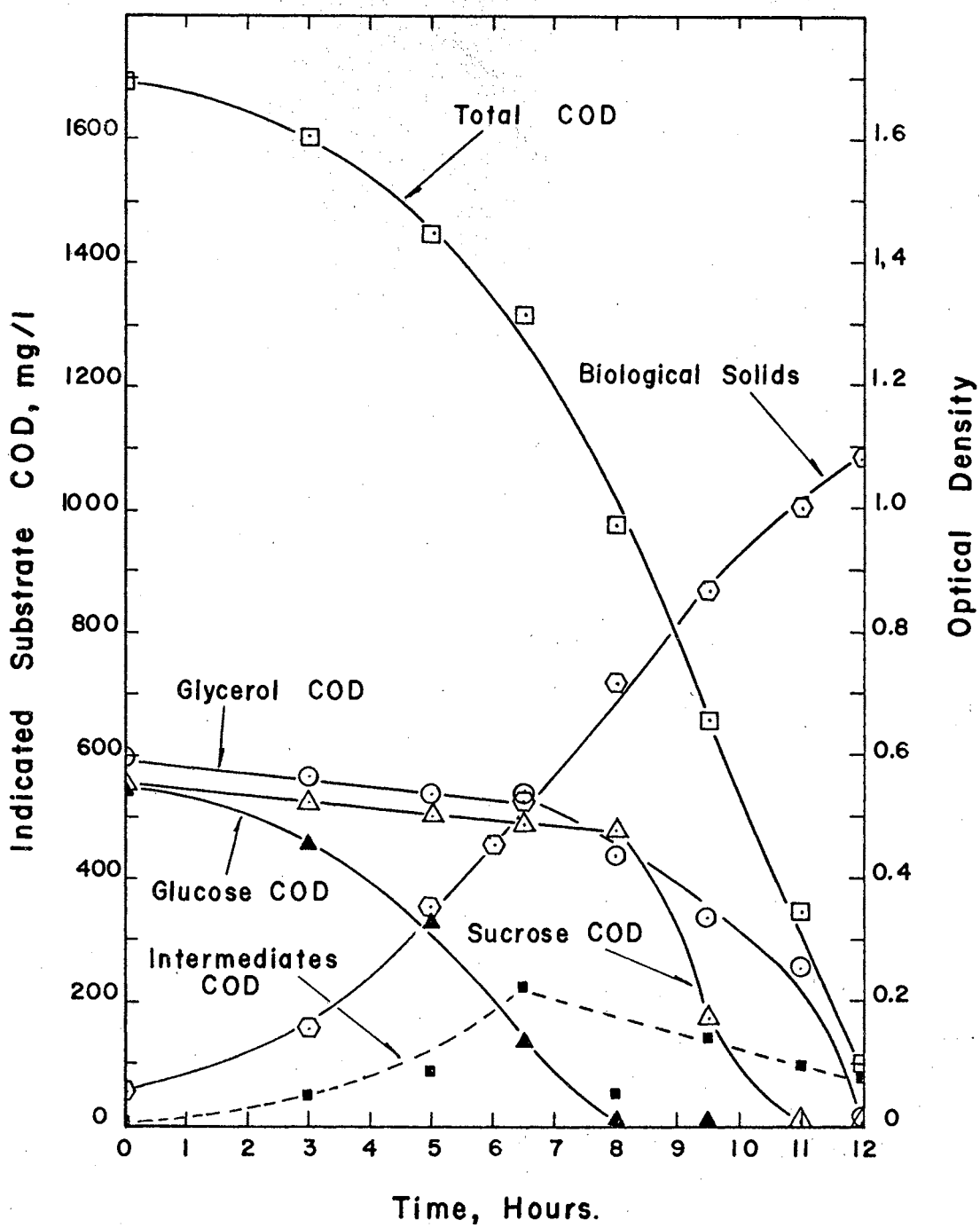

Figure 48 - System performance in the combined unit of glucose, sucrose, and glycerol; young cells acclimated to glycerol.

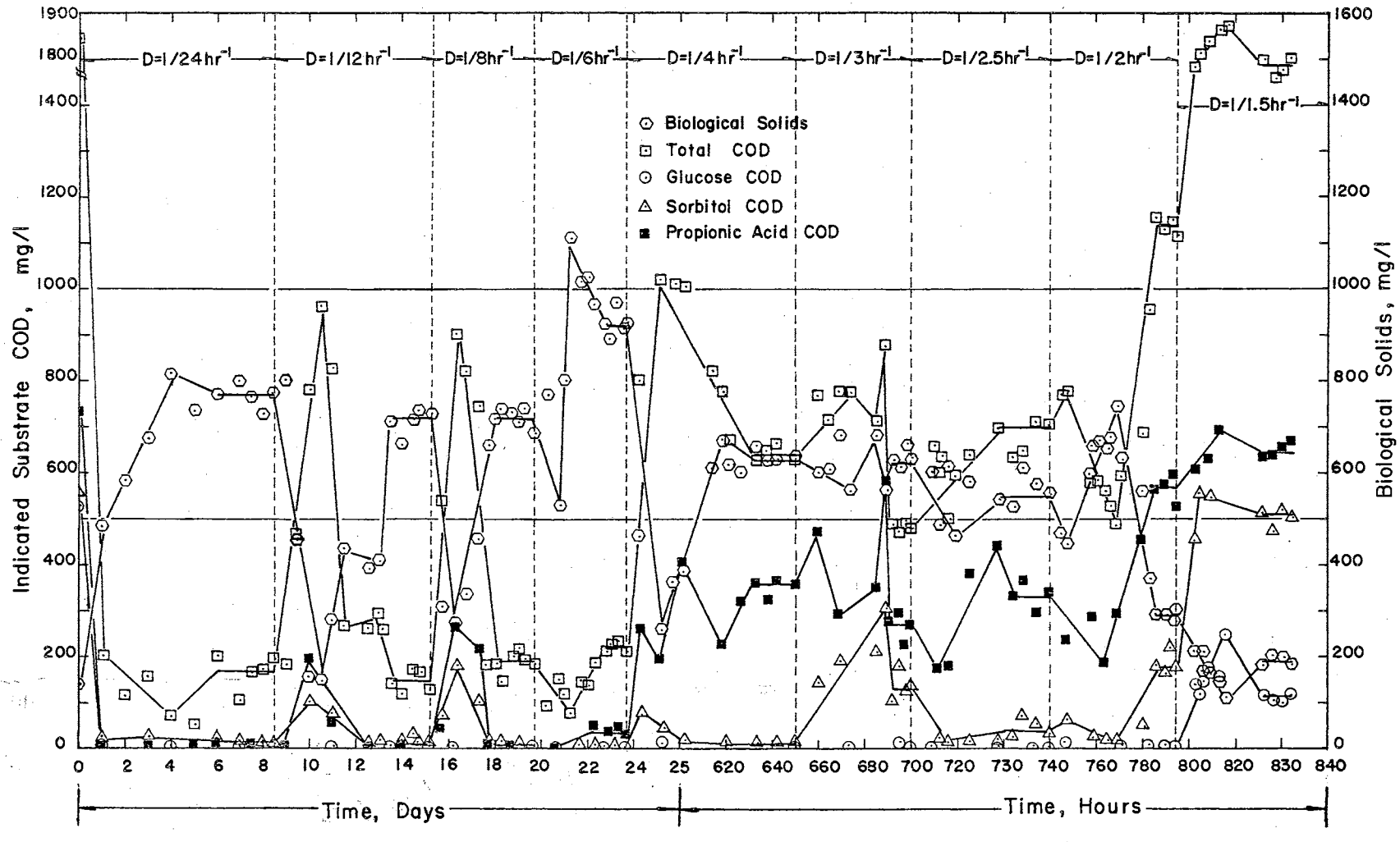

Figure 49 - System performance in the continuous flow activated sludge unit growing on glucose, sorbitol, and propionic acid at various dilution rates.

at the dilution rate of 1/24 hr^{-1}. Within 6 days of operation at this dilution rate, the biological solids rose from 130 mg/l to a steady value of approximately 220 mg/l. During this time only traces of periodate-reactive compound were detected in the effluent. The steady-state cell yield coefficient and system efficiency were determined to be 45 and 91 per cent, respectively.

The efficiency of the system was disrupted severely when the dilution rate was increased from 1/24 to 1/12 hr^{-1}. Within two days after shifting the flow rate, the biological solids dropped rapidly to a minimum level of approximately 150 mg/l, while the total COD rose sharply to nearly 960 mg/l. Thereafter, the effluent COD returned to a new lower steady-state value (approximately 150 mg/l) in response to the recovery in solids concentration to approximately 720 mg/l. A very small portion of this effluent COD was attributable to sorbitol and propionic acid. However, there was considerable leakage in substrate during the transient state; the maximum substrate concentrations amounted to 200 mg/l propionic acid COD and 100 mg/l sorbitol COD. It was observed that the increase in flow rate tended to decrease the cell yield coefficient (Y = 42 per cent) and increase the system efficiency (E = 92 per cent).

Another severe system disruption occurred when the dilution rate was increased to 1/8 hr^{-1}. During the transient, the total COD increased to a peak value of 900 mg/l and was rapidly reduced to a new steady value of 190 mg/l after approximately 2.75 days (8 detention times). The biological solids level dropped to a minimum of 270 mg/l, then increased sharply to the steady-state value of 725 mg/l. It is noted that the order of leakage in this transient was propionic acid >

sorbitol > glucose. There was essentially no leakage of substrate during the steady-state period. The steady-state levels of biological solids concentration and effluent COD indicate that there was no significant change in the cell yield (Y = 43 per cent) and the system efficiency (E = 90 per cent). It should be noted that throughout the operation at these low dilution rates (D = 1/24, 1/12, and 1/8 hr^{-1}) there was no problem with complete mixing in regard to biological solids.

There was very little disruption of substrate removal efficiency when the dilution rate was increased to 1/6 hr^{-1}. Nevertheless, there was considerable fluctuation in biological solids level during the well-defined transient state. It is important to note that the hydraulic shock loading brought about a notable change in microbial predominance and led to incomplete mixing of biological solids. The mixed liquor gradually developed a yellowish hue (from brownish gray) and a loose, rapidly settling floc, which prevented complete mixing in the reactor (e.g., OD_r = 0.921 versus OD_e = 0.745 at 498 hrs). For these reasons, a considerable portion of the solids were accumulated in the reactor (maximum value of 1110 mg/l at 510 hours). The system gradually exhibited better mixing and the solids concentration attained a relatively steady condition (e.g., OD_r = 1.000 versus OD_e = 0.939 at 572 hrs). Data taken during this period (solids 920 mg/l, effluent COD 220 mg/l, propionic acid COD 30 mg/l, and a trace amount of sorbitol) were adjudged to represent "steady-state" behavior at D = 1/6 hr^{-1}.

It is seen that a very severe disruption of substrate removal and a rapid washout of biological solids were caused by the change of dilution rate from 1/6 to 1/4 hr^{-1}. The solids level dropped from approximately 920 mg/l to a minimum of 360 mg/l. There was a corresponding

increase in the effluent COD to 1020 mg/l and then a recovery to approximately 630 mg/l. There was considerable fluctuation in leakage of the specific carbon sources during the transient state. The maximum substrate concentrations in the effluent during this period amounted to 400 mg/l propionic acid COD and 80 mg/l sorbitol COD. At this most severe disruption period, more than half of the effluent COD could be attributed to metabolic intermediates. The steady-state leakages of substrate were somewhat less severe than those observed during the transient state (propionic acid COD 360 mg/l, sorbitol COD 20 mg/l). The system efficiency dropped to 81 per cent, while the sludge yield returned to 42 per cent.

In response to the increase in dilution rate from 1/4 to 1/3 hr^{-1}, there were considerable fluctuations in biological solids concentration, effluent COD and leakage of substrates (sorbitol and propionic acid). During this period of operation there was a distinct change in predominating species as adjudged by the development of a greenish white color in the reactor mixed liquor. Although a few small cell aggregates were being retained in the reactor, at 200 hours the unit had attained a relatively steady-state operation with respect to the levels of solids (620 mg/l) and effluent COD (480 mg/l) and was adjudged to be nearly completely mixed (OD_r = 0.921 versus OD_e = 0.862). Approximately 270 mg/l of the effluent COD was attributable to propionic acid and about 130 mg/l was contributed by sorbitol (periodate-reactive material). It is noted that the steady-state efficiency of substrate removal decreased to 74 per cent but the cell yield coefficient increased to 45 per cent.

The flow rate was next changed to a dilution rate of 1/2.5 hr^{-1}.

The biological solids and effluent COD levels fluctuated mildly in response to the shock. On the whole, the system responded satisfactorily. After the transient (about 30 hours or 12 detention times) was completed, the biological solids level remained fairly constant at approximately 550 mg/l, which indicated a cell yield of 48 per cent. During this period, the reactor exhibited a fairly good degree of complete mixing (e.g., OD_r = 0.886 versus OD_e = 0.824 at 740 hrs). The steady-state effluent COD was approximately 700 mg/l, indicating a low removal efficiency of 62 per cent. The order of substrate leakage was again propionic acid (330 mg/l) > soribtol (40 mg/l) > glucose (traces).

Severe fluctuations in biological solids and effluent COD were observed after increasing the dilution rate to 1/2 hr^{-1}. At first the solids began to wash out, then there was an increase in solids concentration: the COD exhibited a complementary trend. However, after 29 hours (14.5 detention times) the cells again began to wash out and effluent COD increased. It is interesting to note that during the succeeding hours of operation, the mixed liquor developed a deep green color, providing evidence of changes in predominance. After 46 hours (23 detention times) of operation at this dilution rate, it appeared that the system had attained an equilibrium with respect to biological solids concentration (approximately 290 mg/l) and effluent COD (approximately 1130 mg/l) and the system was adjudged to be completely mixed (OD_r = 0.688 versus OD_e = 0.643 at 795 hrs). It is seen that half of the effluent COD was attributable to propionic acid COD (approximately 570 mg/l) and about one-third was sorbitol COD (230 mg/l). On account of this considerable leakage, the system efficiency was only 39 per cent. The steady-state yield was 40 per cent.

When the dilution rate was increased to 1/15 hr^{-1}, most of the propionic acid and sorbitol remained unutilized and a small portion of the glucose appeared in the effluent. The system eventually attained a new steady-state condition with respect to biological solids (approximately 190 mg/l) and effluent COD (approximately 1490 mg/l); the reactor was adjudged to be completely mixed (e.g., OD_r = 0.409 versus OD_e = 0.367 at 832 hrs). The data taken during this period (hour 826 to 832) were used to represent the "steady-state" responses of the system at D = 1/1.5 hr^{-1}.

The average steady-state parameters taken from Figure 49 and production of intermediates at the various dilution rates are shown in Figure 50. All three substrates were utilized completely at dilution rates lower than 1/8 hr^{-1} (0.125 hr^{-1}); thereafter a portion of the propionic acid started to leak from the reactor. The leakage of sorbitol began at a dilution rate of 1/4 hr^{-1}, whereas no glucose was detected in the effluent until the dilution rate was increased to 1/1.5 hr^{-1} (0.667 hr^{-1}). When the system was operated at D = 1/1.5 hr^{-1}, most of the propionic acid COD (740 mg/l), approximately 98 per cent) and sorbitol COD (510 mg/l, approximately 90 per cent) were diluted out, while one-fifth of the glucose (110 mg/l COD) remained unutilized. The results clearly indicate that the order of preferential substrate utilization was glucose > sorbitol > propionic acid. It is discerned that at dilution rates lower than 1/2 hr^{-1}, the increase of total COD leakage was somewhat parallel to the increase of propionic acid leakage. During this period the production of metabolic intermediates increased propotionally with increasing dilution rate. There was a corresponding leakage of glucose when the accumulated intermediates decreased from

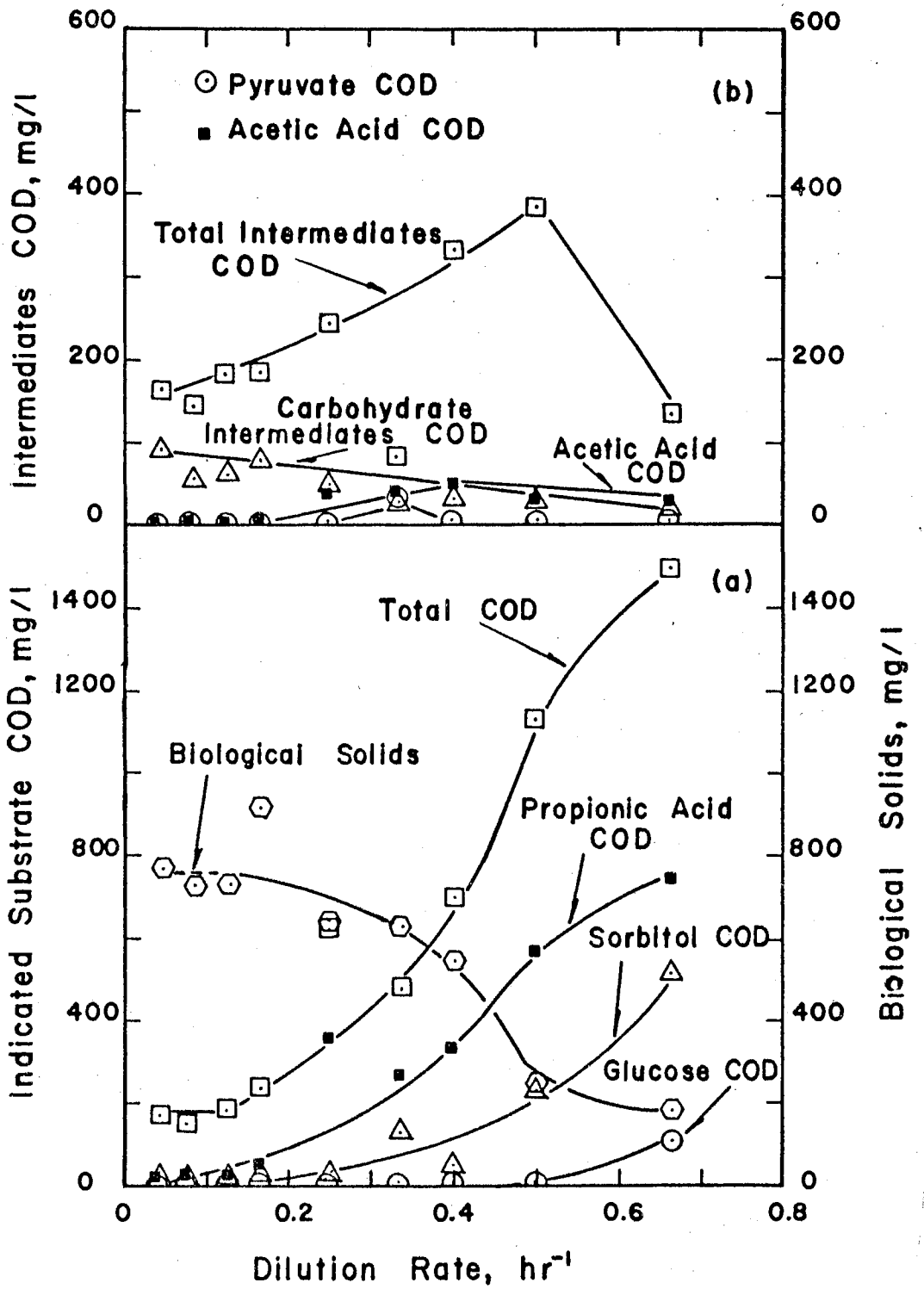

Figure 50 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, sorbitol, and propionic acid at various dilution rates; (b) intermediates accumulation.

approximately 380 mg/l to 130 mg/l. Throughout this series of experiments, only a small portion of the excreted intermediates was attributable to acetic acid (less than 50 mg/l) and pyruvate (less than 30 mg/l). At dilution rates lower than 1/6 hr^{-1} (0.167 hr^{-1}), a somewhat higher amount (approximately 80 mg/l) could be accounted for as carbohydrate; however, the carbohydrate intermediates decreased with increasing dilution rate. Thus only 30 mg/l was identified as carbohydrate when the accumulation of intermediates reached the peak value at $D = 1/2\ hr^{-1}$.

J. Studies on Substrate Removal in a Mixture of Glucose, Glycerol, and Butyric Acid by a Heterogeneous Population (Continuous Flow Reactor)

The metabolic responses to the combined glucose-glycerol-butyric acid substrate during growth in the continuous flow reactor at various dilution rates are shown in Figure 51. Both glucose and butyric acid were consumed within a half-day's operation, but no significant removal of glycerol occurred during this period. It may be possible that the 610 mg/l glycerol COD at 0.5 day was not the original feed glycerol; it might be accounted for as periodate-reactive intermediates produced from metabolism of one or more of the substrates. The considerable decrease in glycerol COD (500 mg/l) from 0.5 to 1.0 day and the corresponding slight changes in solids concentrations and total COD might indicate that during this period a large portion (420 mg/l) of the periodate-reactive material was converted to intermediates non-reactive to periodate. After the initial removal of substrates, there was a severe disruption of substrate removal, and the order of leakage was butyric acid (560 mg/l) > glycerol (300 mg/l) > glucose (traces). Thereafter, the system attained a highly efficient (92 per cent) steady-state

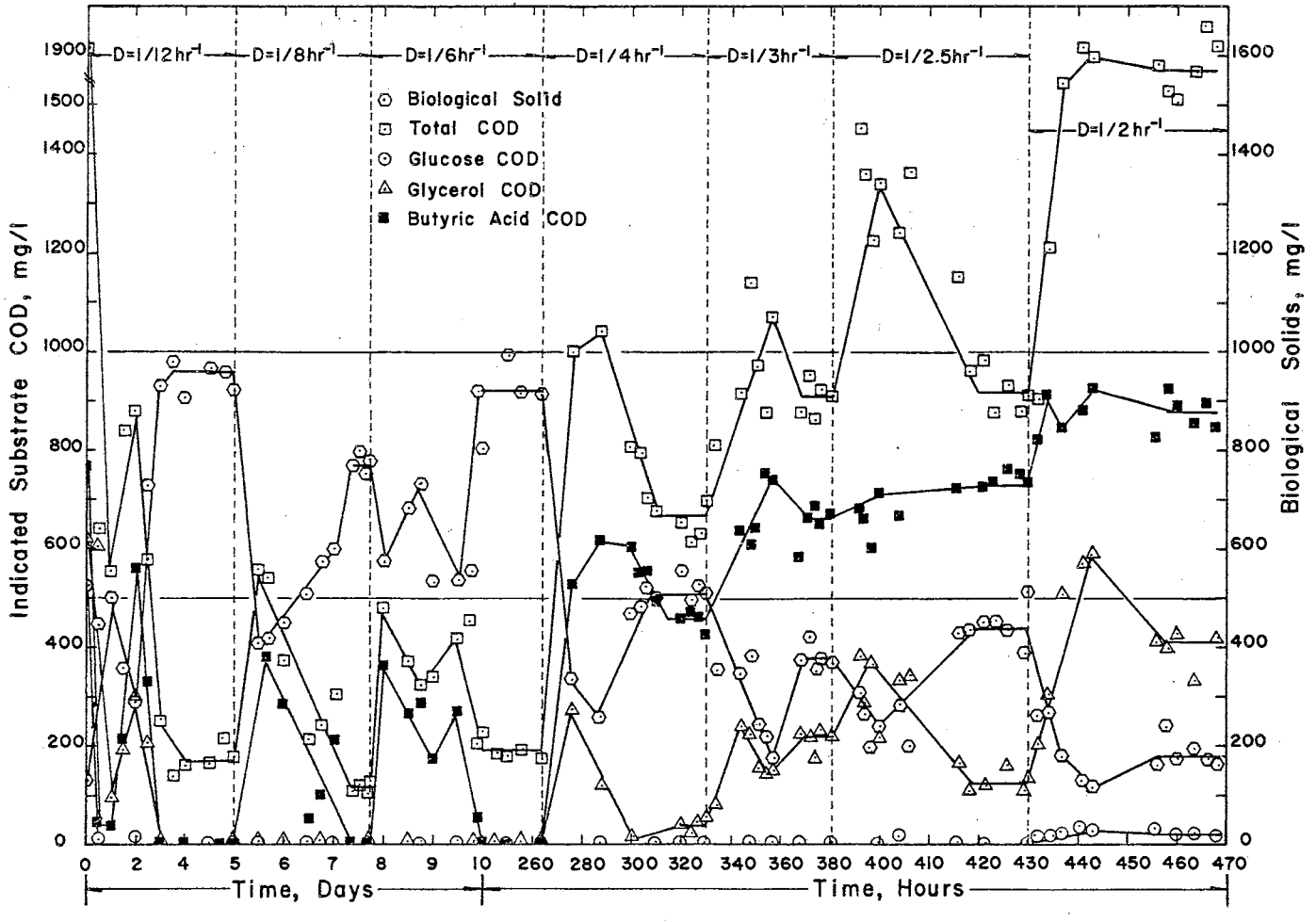

Figure 51 - System performance in the continuous flow activated sludge unit growing on glucose, glycerol, and butyric acid at various dilution rates.

condition. The steady-state solids concentration (approximately 960 mg/l) and the effluent COD (approximately 170 mg/l) indicate a cell yield of 52 per cent. During this period the system was adjudged to have a fairly good degree of complete mixing (e.g., OD_e = 0.959 versus OD_r = 1.022 at 5.0 days).

A severe disruption of substrate removal and a noticeable change in predominating species were brought about after the dilution rate was changed to 1/8 hr^{-1}. Within 0.5 day of operation after increasing the dilution rate, the biological solids dropped to 400 mg/l while the effluent COD and butyric acid leakage rose to 560 mg/l and 400 mg/l, respectively. During cell washout, the color of the mixed liquor changed from milky white to light green. The system then recovered, and 2.5 days (7.5 detention times) after applying the hydraulic shock a new steady-state condition was attained. At this time the biological solids concentration was approximately 770 mg/l, and the effluent COD dropped to 120 mg/l. The high system efficiency E = 94 per cent) and the relatively low cell yield (Y = 41 per cent) might be attributable in small part to incomplete mixing of the reactor (e.g., OD_r = 1.046 versus OD_e = 0.921 at 7.75 days).

A change in dilution rate from 1/8 to 1/6 hr^{-1} caused considerable transient disturbance to the system. During the transient period there was a considerable fluctuation in the effluent concentrations of total COD and butyric acid (the peak leakages were 480 mg/l and 360 mg/l, respectively). Within approximately 2.25 days (9 detention times) the system exhibited a fairly constant behavior. The biological solids recovered to approximately 920 mg/l (Y = 51 per cent) while effluent COD returned to approximately 10 mg/l (E = 91 per cent). Throughout

operation at D = 1/6 hr^{-1}, no significant amount of glucose or glycerol was found in the effluent. It was noted that the system at this dilution rate was more completely mixed (e.g., OD_r = 1.034 versus OD_e = 0.959 at 11 days) than it had been at D = 1/8 hr^{-1} and that the green color of the mixed liquor was deeper than during growth at the previous dilution rate.

When the dilution rate was increased to 1/4 hr^{-1}, a very severe transient disruption of treatment efficiency ensued. The biological solids were rapidly washed out after the increase in dilution rate, and attained a new fairly constant level, approximately 510 mg/l (Y = 38 per cent) in 45 hours (11 detention times). During this period the effluent COD rose to 1040 mg/l and then dropped to a steady-state level of approximately 670 mg/l (E = 65 per cent). The maximum substrate leakages during the transient were 610 mg/l butyric acid and 270 mg/l glycerol, whereas the approximate steady-state leakages were 460 mg/l butyric acid and 40 mg/l glycerol. The system exhibited a fairly good degree of complete mixing (OD_r = 0.763 versus OD_e = 0.710 at 330 hours) and the color of the mixed liquor returned to light green.

When the dilution rate was increased to 1/3 hr^{-1}, a well-defined transient pattern was observed; then the system returned to a new steady-state. At the time of severest disruption, more than 1050 mg/l COD (about 750 mg/l butyric acid and 200 mg/l glycerol) was found in the reactor effluent, and the biological solids level decreased to 180 mg/l. About 40 hours after the shift of dilution rate (13 detention times), the system exhibited a steady-state behavior. During this period there was a considerable leakage of COD (approximately 910 mg/l) which included 660 mg/l butyric acid COD and 220 mg/l glycerol COD),

and the solids level in the reactor was approximately 380 mg/l. The cell yield was only 35 per cent, and the treatment efficiency was 55 per cent. Thus it appeared that the system was beginning the dilute-out phase. Throughout the experiment at D = 1/3 hr^{-1}, there was no problem with complete mixing in regard to biological solids (e.g., OD_r = 0.620 versus OD_e = 0.585 at 581 hours).

When the dilution rate was increased to 1/2.5 hr^{-1}, a very severe transient disruption of treatment efficiency occurred and a very noticeable change in microbial predominance was brought about by this shock load. During the 38-hour transient period (15 detention times) the effluent leakages amounted to 1370 mg/l total COD, 710 mg/l butyric acid COD, and 380 mg/l glycerol COD, while the biological solids concentration reached 200 mg/l. Although the reactor mixed liquor gradually developed a few loosely bound greenish-white flocs, the system exhibited a fairly good degree of complete mixing (e.g., OD_r = 0.699 versus OD_e = 0.643 at 415 hours). Under steady-state conditions, the approximate values of the system parameters were: total COD leakage 920 mg/l (butyric acid COD 730 mg/l, glycerol COD 130 mg/l, and trace amounts of glucose), biological solids 440 mg/l, cell yield 40 per cent, and system efficiency 54 per cent.

The highest dilution rate applied to this system was D = 1/2 hr^{-1}. The system was severely disrupted by this hydraulic shock load. There is little doubt that the system was operating close to the maximum growth rate, though a fairly constant steady-state was attained within 28 hours (14 detention times). As for the previous dilution rate, the order of steady-state substrate leakages was butyric acid (approximately 880 mg/l) > glycerol (410 mg/l) > glucose (20 mg/l). This substantial

leakage (1570 mg/l total COD) indicates that only 22 per cent of the carbon source was removed at D = 1/2 hr^{-1}. The steady-state solids concentration was approximately 180 mg/l, which provided a cell yield of approximately 42 per cent.

The approximate values of the various steady-state parameters taken from Figure 51 for various dilution rates are given in Figure 52a. It is noted that the microbial population completely utilized all of the carbon sources at dilution rates lower than 0.167 hr^{-1} (1/6 hr^{-1}). Glucose was the preferred carbon source in this combined system, and was nearly completely consumed even at a dilution rate as high as 0.5 hr^{-1} (1/2 hr^{-1}). Glycerol was the second most easily used carbon source and it began to appear in the effluent at D = 0.25 hr^{-1}; thereafter, glycerol leakage gradually increased with increasing dilution rate. As in the case of glycerol, butyric acid leakage started at D = 0.25 hr^{-1} and increased with increasing dilution rate. However, the amount of butyric acid leakage was much more than that of glycerol. At the termination of this experiment, 880 mg/l of butyric acid COD (about 98 per cent) and 410 mg/l glycerol COD (approximately 67 per cent) remained unutilized. All of these results clearly indicate that the order of priority of substrate removal was glucose > glycerol > butyric acid, regardless of various changes in microbial predominance which were observed during the experiment. It is important to note that the removal sequence observed from various steady-states was consistent with that of the various transient states shown in Figure 51.

The metabolic intermediate curves (Figure 52b) indicate that throughout this series of experiments, substantial amounts of intermediates were not accumulated. The maximum production of intermediates

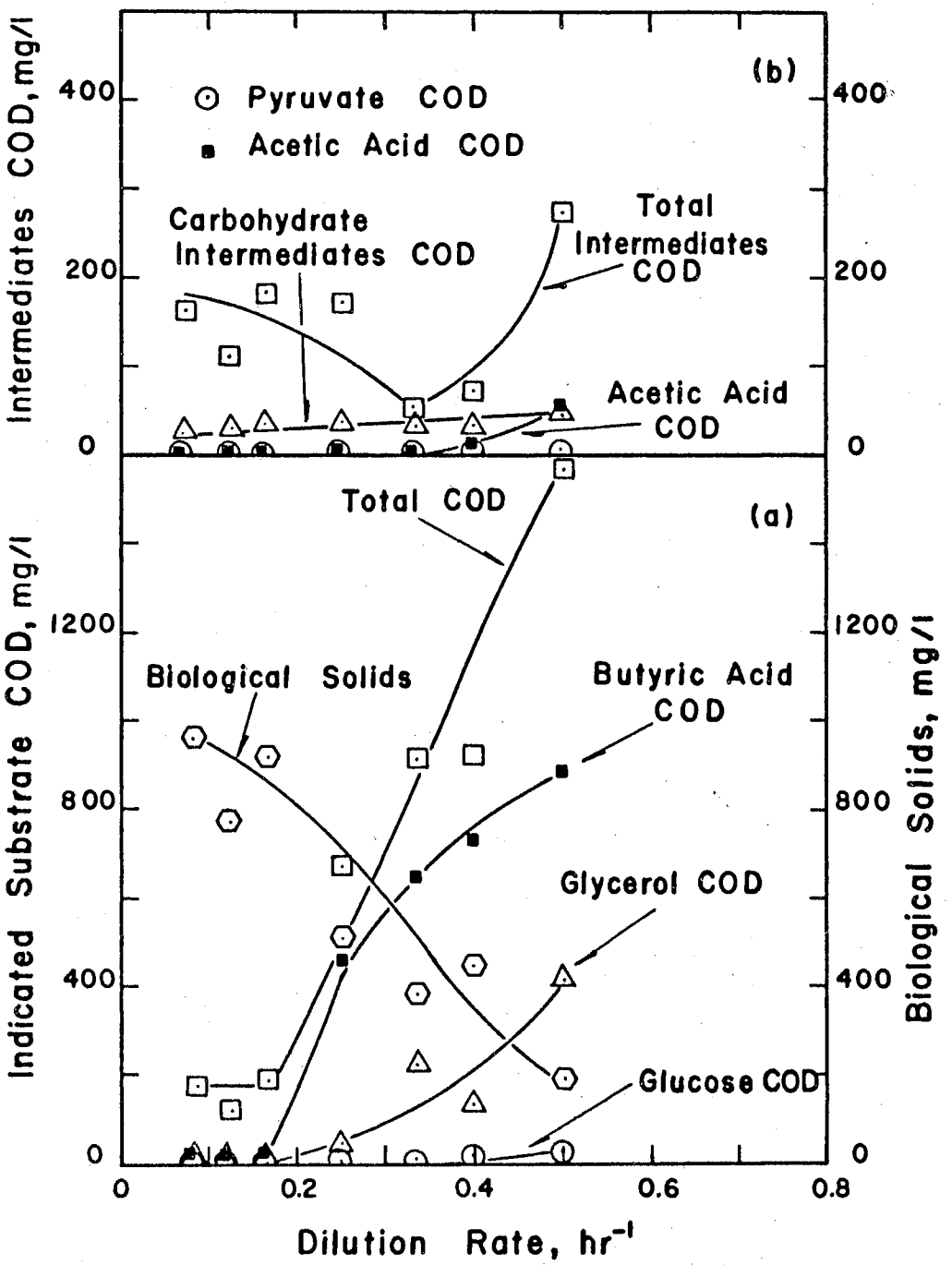

Figure 52 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, glycerol, and butyric acid at various dilution rates; (b) intermediates accumulation.

(approximately 270 mg/l) occurred at D = 1/2 hr^{-1} and only a small portion of these were accounted for as carbohydrate (40 mg/l) and acetic acid (50 mg/l). The concentration of carbohydrate intermediates (anthrone-reactive materials) was fairly constant throughout the experiment, but acetic acid was found only at D = 1/2 hr^{-1}. No significant excretion of pyruvate was detected in this system. A minimum accumulation of intermediates (50 mg/l) was found at D = 0.333 hr^{-1} (1/3 hr^{-1}).

K. Studies on Substrate Removals in a Mixture of Glucose, Galactose, Sorbitol and Xylose by a Heterogeneous Microbial Population

I. Batch Experiments

a. Glucose-acclimated Cells

Figure 53 shows the metabolic responses for the control systems. The significant lag period for xylose and galactose metabolism indicates that the glucose-acclimated sludge did not contain enzyme systems for metabolism of these two substrates at the initiation of the experiment. A very short lag period on sorbitol was observed, as evidenced by the growth pattern on sorbitol in Figure 53 and by semilogarithmic plots of OD versus time for the sorbitol control. This indicates that the sorbitol-degrading enzyme system was very easily induced in the glucose-acclimated sludge or was constitutive in a significant number of species in the initial seed. It is noted that the microbial population grew on glucose (RT = 6.0 hrs) and sorbitol (RT = 10.6 hrs) more rapidly than on galactose (RT = 16.2 hrs) or xylose (RT = 21.6 hrs). Based upon growth rate, acclimation requirement, and accumulation of intermediates, the results for the control units indicate that glucose and sorbitol possessed a rather high potential to interfere with removal of the

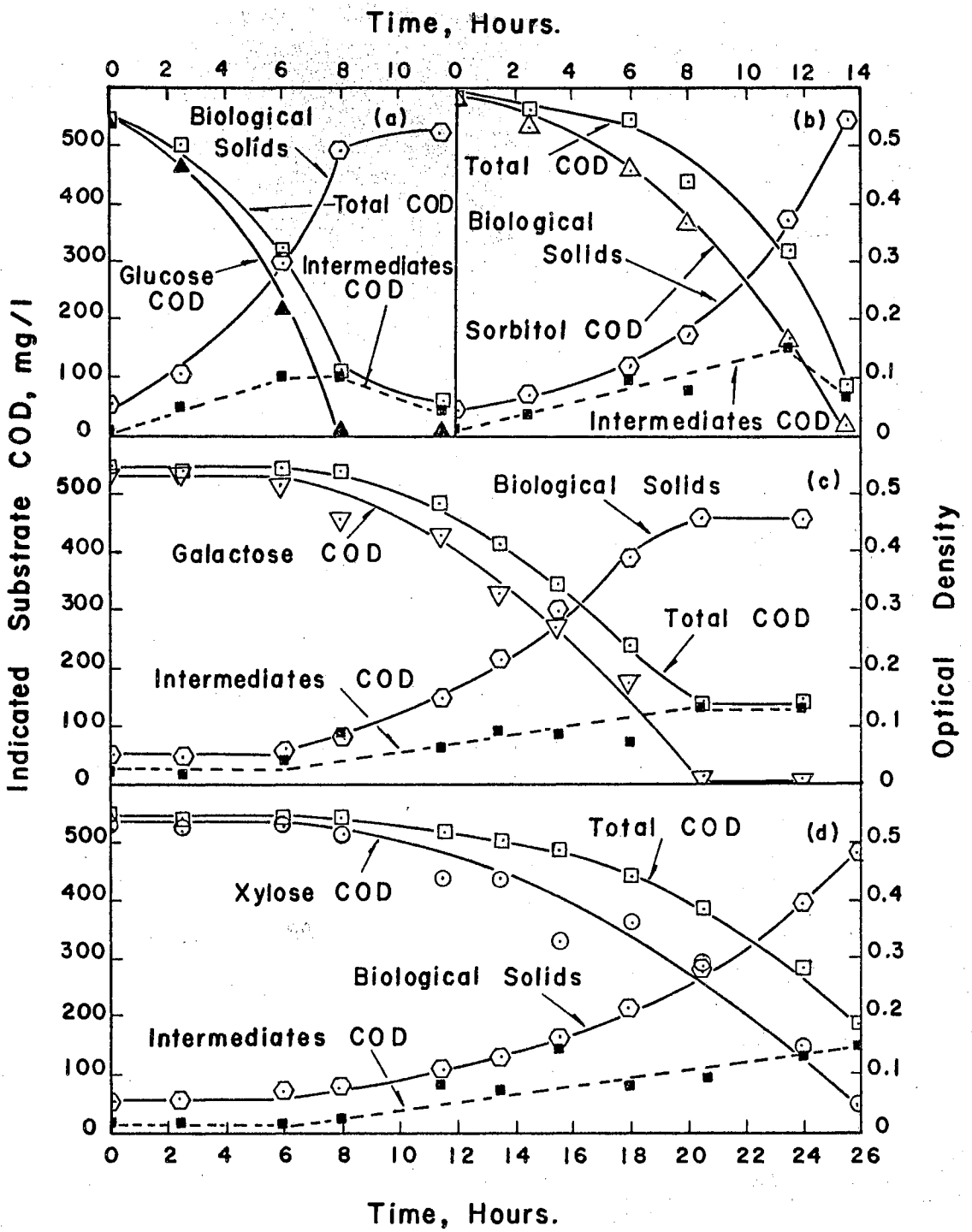

Figure 53 - System performance in the control units of (a) glucose, (b) sorbitol, (c) galactose, and (d) xylose; young cells acclimated to glucose.

other two carbon sources.

It is interesting to note that when these four substrates were used as combined carbon source (Figure 54), a severe disruption of substrate removal was brought about, as evidenced by the pronounced lag period. It appeared that the microbial population was upset by feeding of the mixed substrate and did not use any of the component carbon sources, even glucose, for growth within 2.5 hours of operation. A comparison of reference time for glucose removal in the combined (RT = 13.4 hrs) and the control (RT = 6.4 hrs) systems also indicates that glucose metabolism was severely disrupted by the presence of the other three substrates. The lags in galactose removal (about 8 hours) and xylose removal (about 11.5 hours) were significantly longer than those in the respective controls, indicating that the synthesis of enzyme systems required for metabolism of these two sugars was retarded by the presence of the other substrates. The long (11.5 hour) blockage of sorbitol removal suggests that the presence of other substrates severely suppressed the catabolism of sorbitol. Comparison of substrate removals in the combined reactor with those in the respective controls reveals that metabolism of both glucose (from RT = 6.4 hrs to RT = 13.4 hrs) and sorbitol (from RT = 11.0 hrs to more than 20 hrs) was greatly retarded, while the metabolism of galactose (from RT = 16.8 to 14.9 hrs) and xylose (from RT = 22.0 to 17.0 hrs) were slightly enhanced in the presence of other substrates. The patterns for removal of these four substrates provide a clear indication that the order of removal was glucose > galactose > xylose > sorbitol. The peak accumulation of intermediates (about 400 mg/l) occurred when glucose was exhausted. Thereafter, only a small portion of the accumulated intermediates

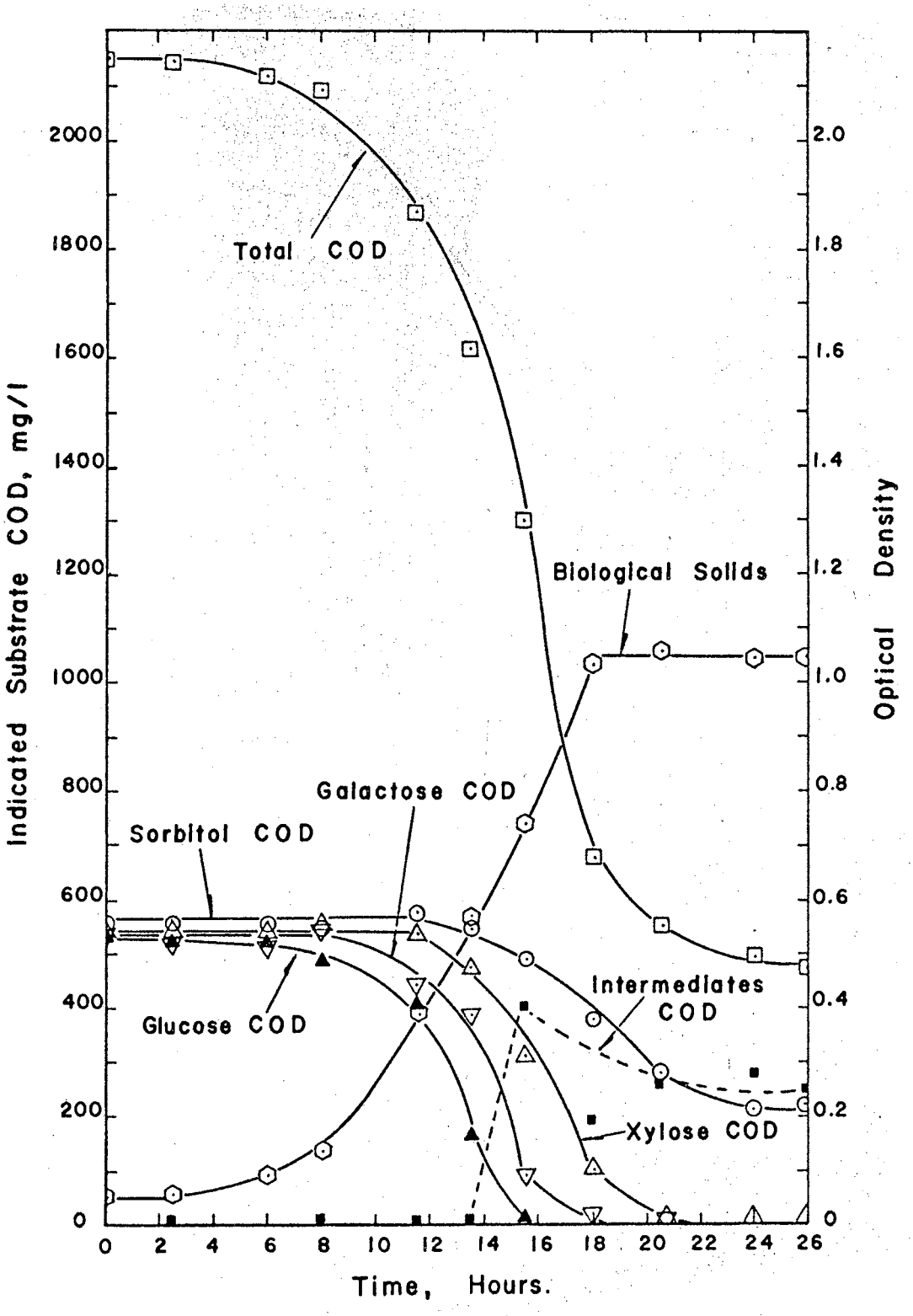

Figure 54 - System performance in the combined unit of glucose, galactose, sorbitol, and xylose; young cells acclimated to glucose.

(about 1/3) was removed; thus approximately 250 mg/l of the accumulated intermediates remained in the reactor at the termination of the experiment. At this time more than 200 mg/l of periodate-reactive COD was found in the reactor.

b. Xylose-acclimated Cells

The results from the control units (Figure 55) indicate that the xylose-acclimated cells needed no acclimation for growth on glucose or sorbitol, but required a significant acclimation to galactose. The organisms grew on glucose (RT = 8.0 hrs), xylose (RT = 10 hrs), and sorbitol (RT = 11.2 hrs) more rapidly than on galactose (RT = 16.8 hrs). From these responses and the accumulation of intermediates (dashed curves), it seems that glucose and xylose possessed a potential capability of interfering with removal of the other two carbon sources.

As in the experiment with glucose-acclimated cells, these xylose-grown cells exhibited a considerable lag period (about 9 hrs) before growing on the mixed substrate (Figure 56). The sequence of removal in this system was similar to that shown in Figure 54, i.e., glucose > galactose > xylose > sorbitol. The removal of these four substrates was retarded to a significant extent as compared with the respective rates of removal in the controls (glucose from RT = 7.8 to 15 hrs; galactose from RT = 17.2 to 22.8 hrs; xylose from RT = 8.8 to 24.8 hrs, and sorbitol from RT = 11.8 to 26 hrs). Since 9 hours of acclimation were required in the combined unit, the 6-hour delay in galactose enzyme synthesis (from 6 hrs in the control to 12 hrs in the combined system) does not provide clear indication of interference with enzyme synthesis. The removals of sorbitol and xylose did not start until glucose had been exhausted, indicating that enzymes responsible for catabolism of

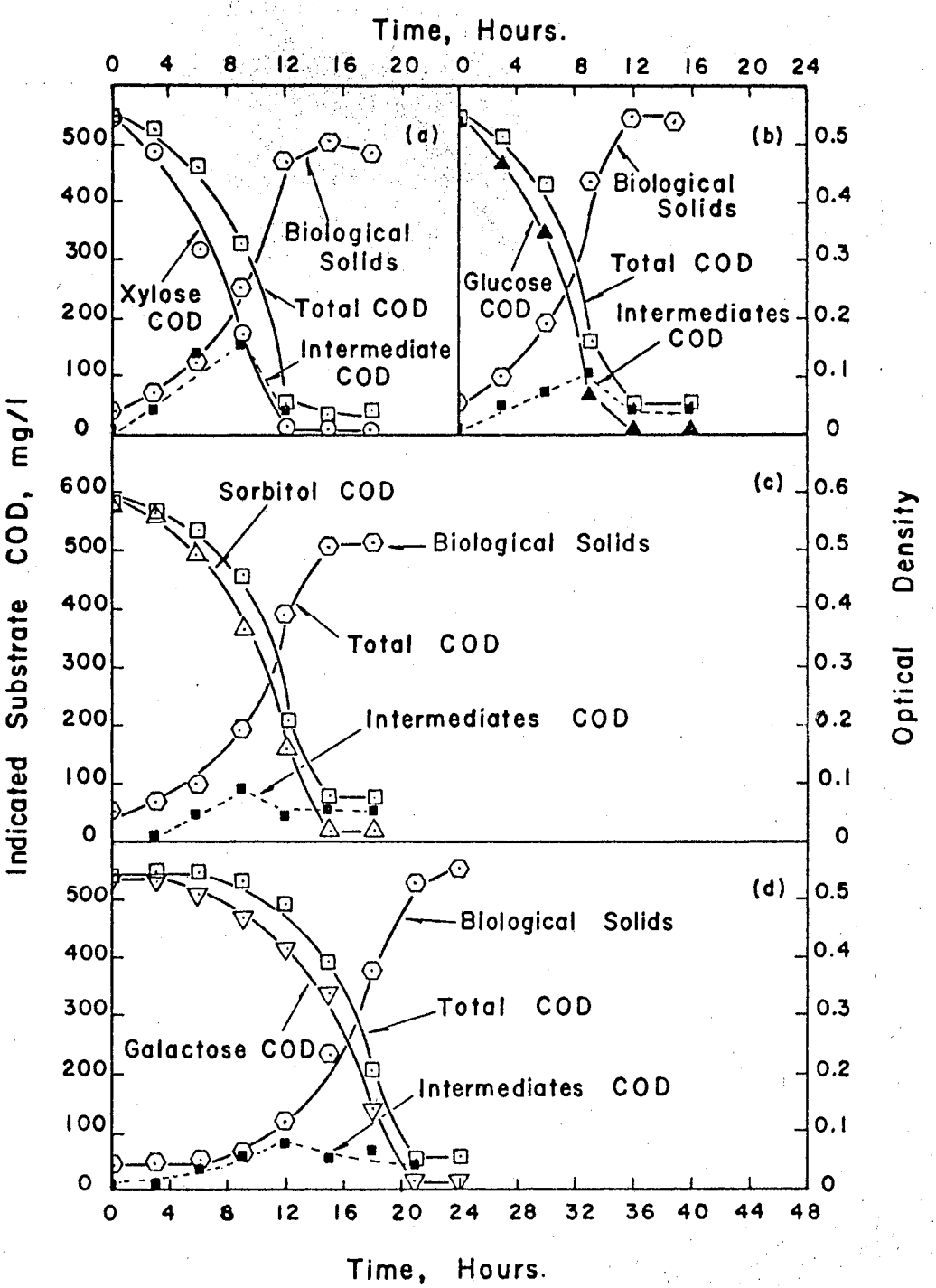

Figure 55 - System performance in the control units of (a) xylose, (b) glucose, (c) sorbitol, and (d) galactose; young cells acclimated to xylose.

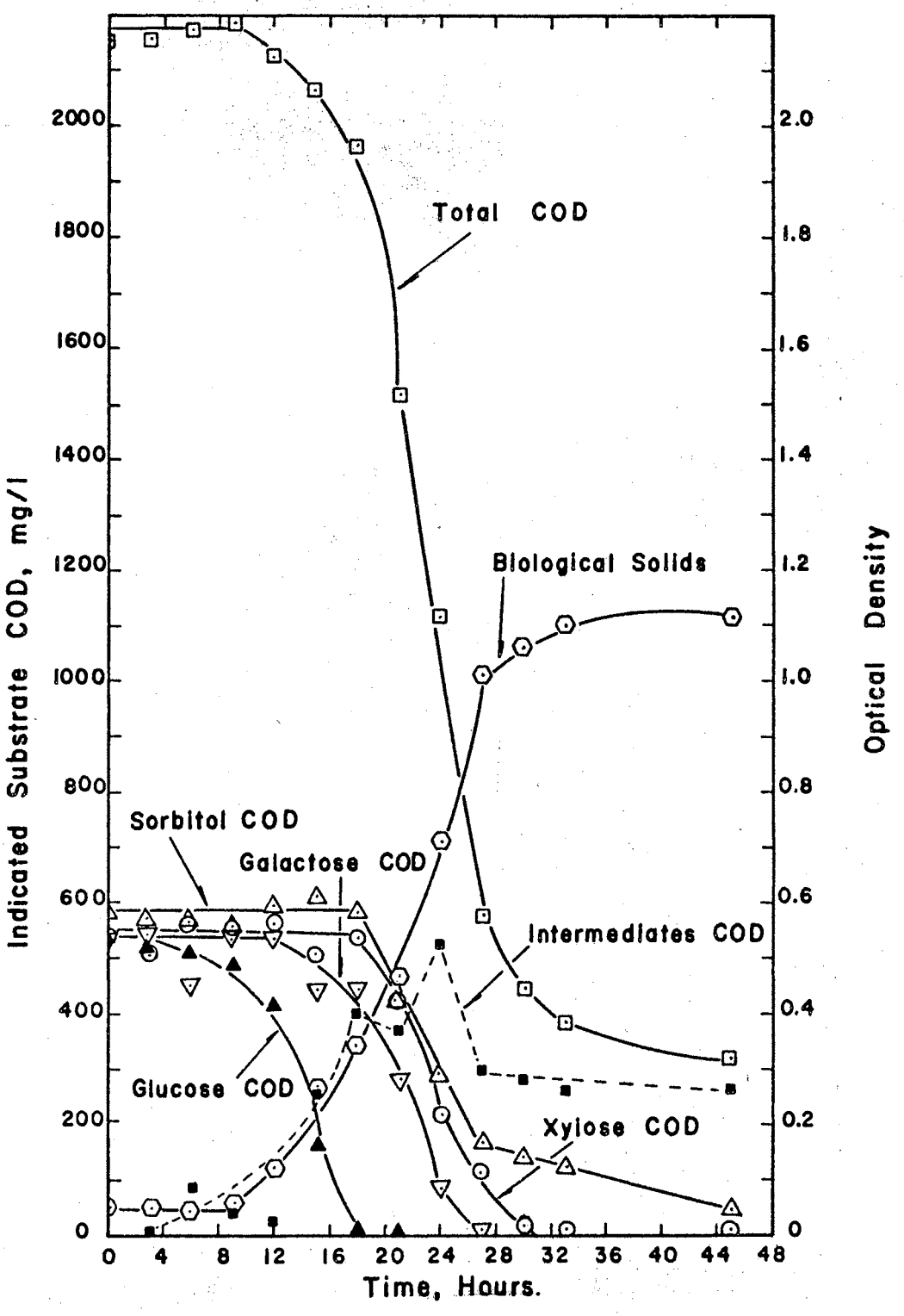

Figure 56 - System performance in the combined unit of glucose, galactose, xylose, and sorbitol; young cells acclimated to xylose.

these two substrates were severely inhibited by glucose. A considerable amount of accumulated intermediates was found during the course of substrate metabolism, (e.g., approximately 400 mg/l at 18 hrs and 530 mg/l at 24 hrs); a large portion of these intermediates remained in the reactor at the termination of the experiment. Thus the treatment efficiency was about 85 per cent.

c. Galactose-acclimated Cells

The growth responses for the control units (Figure 57) indicate that the galactose-acclimated cells contained enzymes for glucose catabolism but required significant lag periods in order to grow on xylose (about 14 hours) and sorbitol (about 8 hrs). The organisms grew on glucose (RT = 6.0 hrs) and galactose (RT = 7.8 hrs) more rapidly than on sorbitol (RT = 15.0 hrs) or xylose (RT = 33.6 hrs, and there was some accumulation of metabolic intermediates in all of the units (galactose > xylose > glucose > sorbitol). All of these results suggest that glucose and galactose exhibited a high potential for interference with the removal of the other two substrates.

In the combined system (Figure 58), the cells initiated growth immediately. Glucose and galactose were removed concurrently without a significant lag period; however, glucose removal was slightly affected by the presence of the other substrates (RT = 6.6 hrs in the control versus 7.4 hrs in the combined system), while galactose removal was considerably retarded (from RT = 7.2 hrs to 11.6 hrs). The approximate lag periods for utilization of sorbitol and xylose were 8 hours and 15 hours, respectively. These results indicate that the presence of the other two substrates did not affect the synthesis of enzymes for these substrates to a considerable extent, since the lag periods were 8 hrs

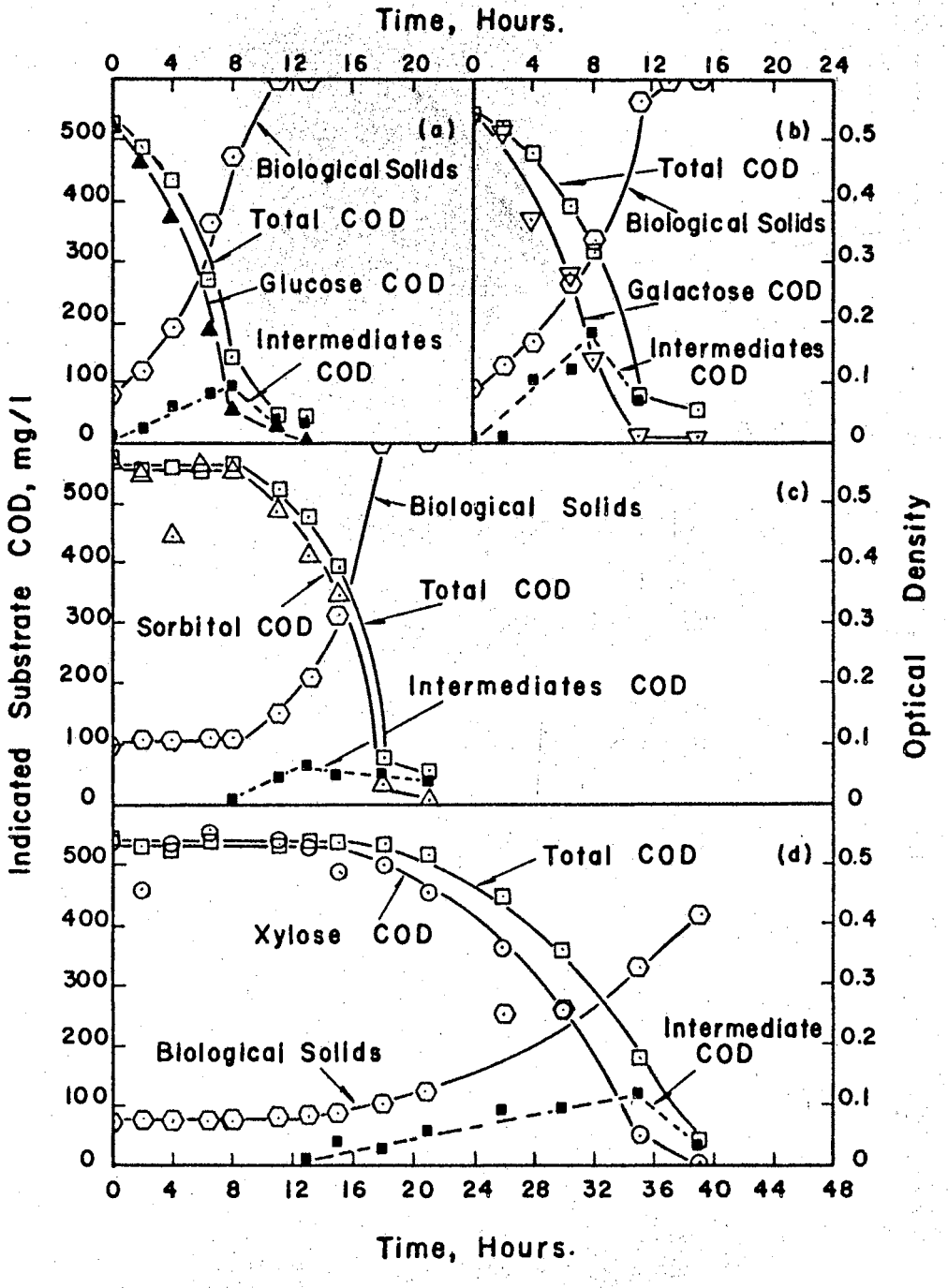

Figure 57 - System performance in the control units of (a) glucose, (b) galactose, (c) sorbitol, and (d) xylose; young cells acclimated to galactose.

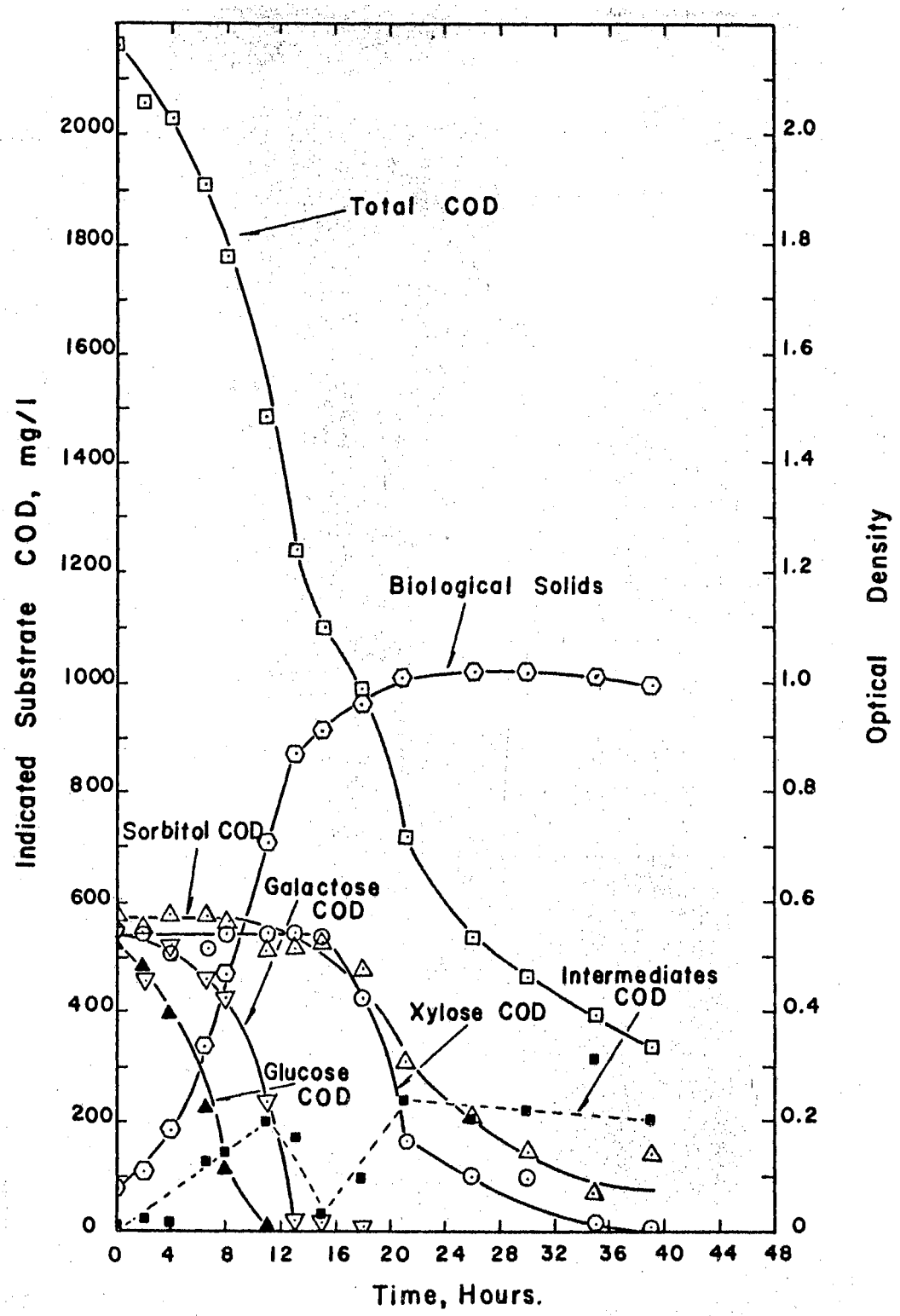

Figure 58 - System performance in the combined unit of glucose, galactose, sorbitol, and xylose; young cells acclimated to galactose.

and 14 hrs in the controls. Although the lag period for xylose was much longer than that for sorbitol, xylose was removed (RT = 21 hrs) more rapidly than was sorbitol (RT = 26 hrs). At the end of the experiment (39 hours) there were 330 mg/l of residual COD in the reactor, yielding a treatment efficiency of 85 per cent.

d. Sorbitol-acclimated Cells

The results for the single substrate units (Figure 59) indicate that the sorbitol-acclimated cells required approximately 8 hours' acclimation to galactose and xylose, and that the cells grew on glucose (RT = 6.2 hrs) and sorbitol (RT = 7.6 hrs) more rapidly than on galactose (RT = 16.2 hrs) and xylose (RT = 21 hrs). Somewhat greater amounts of metabolic intermediates were accumulated from glucose and xylose (about 180 mg/l each) than from sorbitol and galactose (less than 100 mg/l).

As in Figures 54 and 56, there was a significant lag period (about 6 hrs) before the cells grew in the mixed substrate system (Figure 60). During this lag period, it appeared that a small amount of glucose (approximately 80 mg/l) and sorbitol (approximately 40 mg/l) were removed; however, none of the COD was removed from the system during the first 5 hours. Although glucose and sorbitol were removed concurrently from the beginning of the experiment, their removals were considerably retarded (RT_g = 5.6 hrs and RT_s = 8.6 hrs in the controls versus RT_g = 12 hrs and RT_s = 28 hrs in the combined system). The lag period for galactose removal in the combined system (10.5 hrs) was slightly longer than that in the control (about 8 hrs). The lag periods for removal of xylose (8 hrs in the control versus 14 hrs in the combined), and galactose (8 hrs in the control versus 10.5 hrs in the combined) indicate

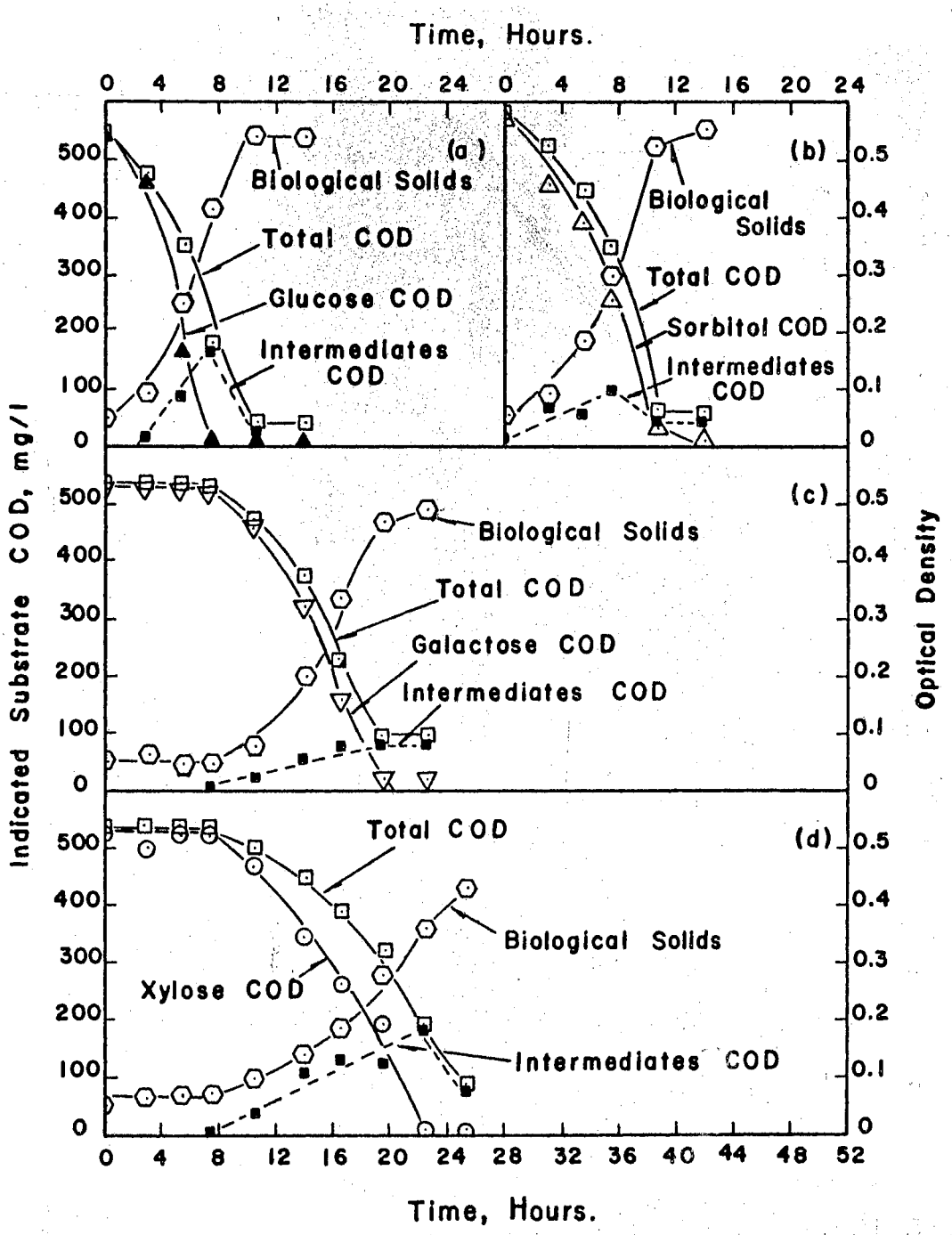

Figure 59 - System performance in the control units of (a) glucose, (b) sorbitol, (c) galactose, and (d) xylose; young cells acclimated to sorbitol.

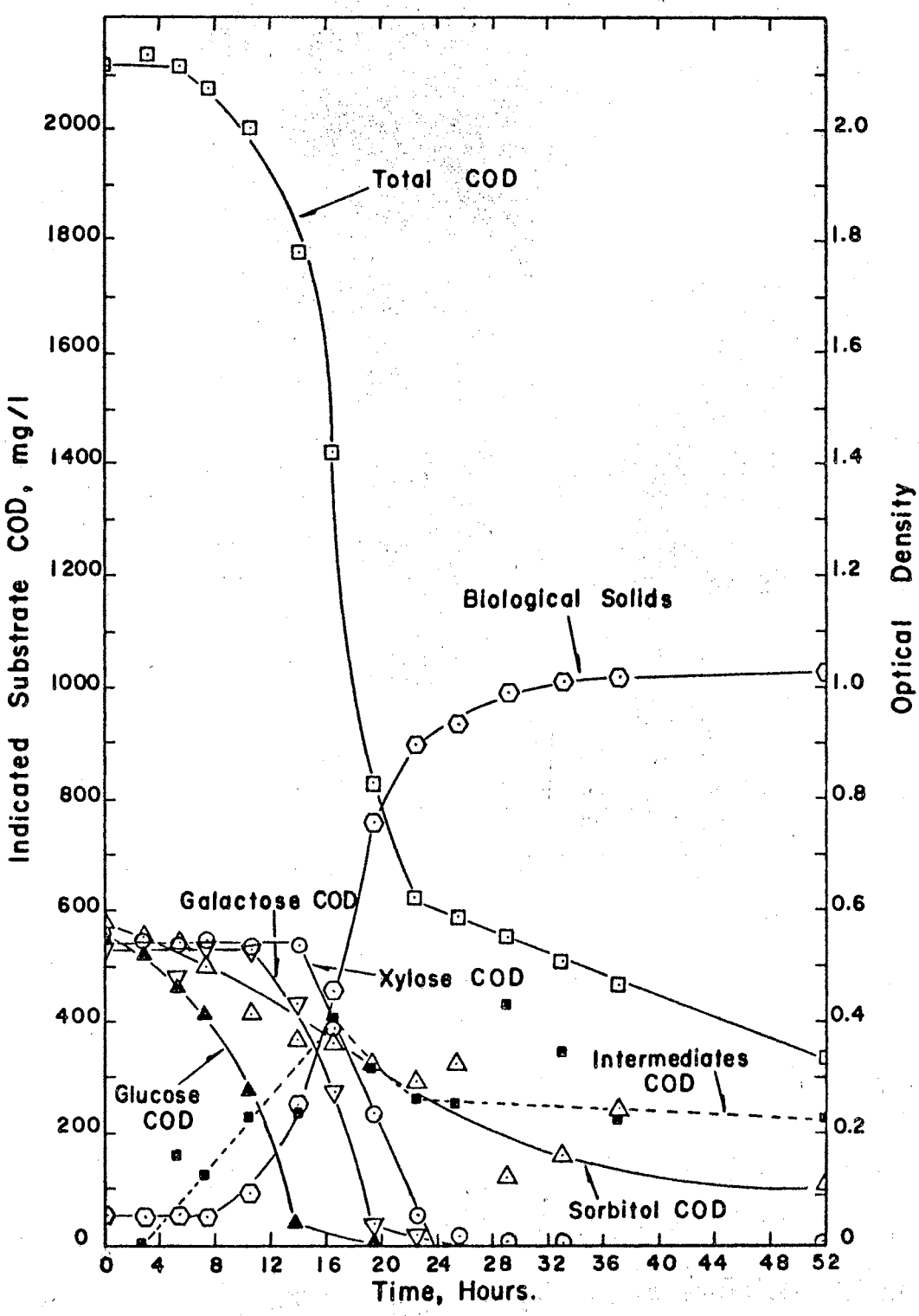

Figure 60 - System performance in the combined unit of glucose, galactose, sorbitol, and xylose; young cells acclimated to sorbitol.

that synthesis of the requisite enzyme systems was retarded due to the presence of the other substrates. The rather slow sorbitol removal (RT = 28 hrs), the considerable fluctuation in sorbitol concentration throughout the experiment, and the presence of 100 mg/l sorbitol COD in the reactor at the termination of the experiment might be attributable in part to the excretion of periodate-reactive material from the metabolism of the other substrates, i.e., the material measured as sorbitol might actually be another compound or compounds. The 330 mg/l residual COD indicates that the treatment efficiency was approximately 84 per cent (compared with 78 per cent in Figure 54, and 85 per cent in Figures 56 and 58.

II. Continuous Flow Experiments

The metabolic responses for the continuous flow system (Figure 61) indicate that glucose was readily metabolized by the microbial population, while galactose, xylose and sorbitol were removed more slowly when the experiment was initiated at $D = 1/12\ hr^{-1}$. During the transient period, the sequence of substrate leakage was sorbitol > xylose > galactose > glucose, which was in accordance with the results of batch experiments. About 3 days after the initiation of this experiment (6 detention times), the system attained a steady-state. At this time, the COD leakage was approximately 230 mg/l (only trace amounts of xylose and sorbitol were identified) yielding a treatment efficiency of 89 per cent. The biological solids concentration was approximately 735 mg/l (Y = 38 per cent). It is important to note that throughout the experiment from $D = 1/12\ hr^{-1}$ to $D = 1/2.5\ hr^{-1}$, there was no problem with complete mixing in regard to biological solids.

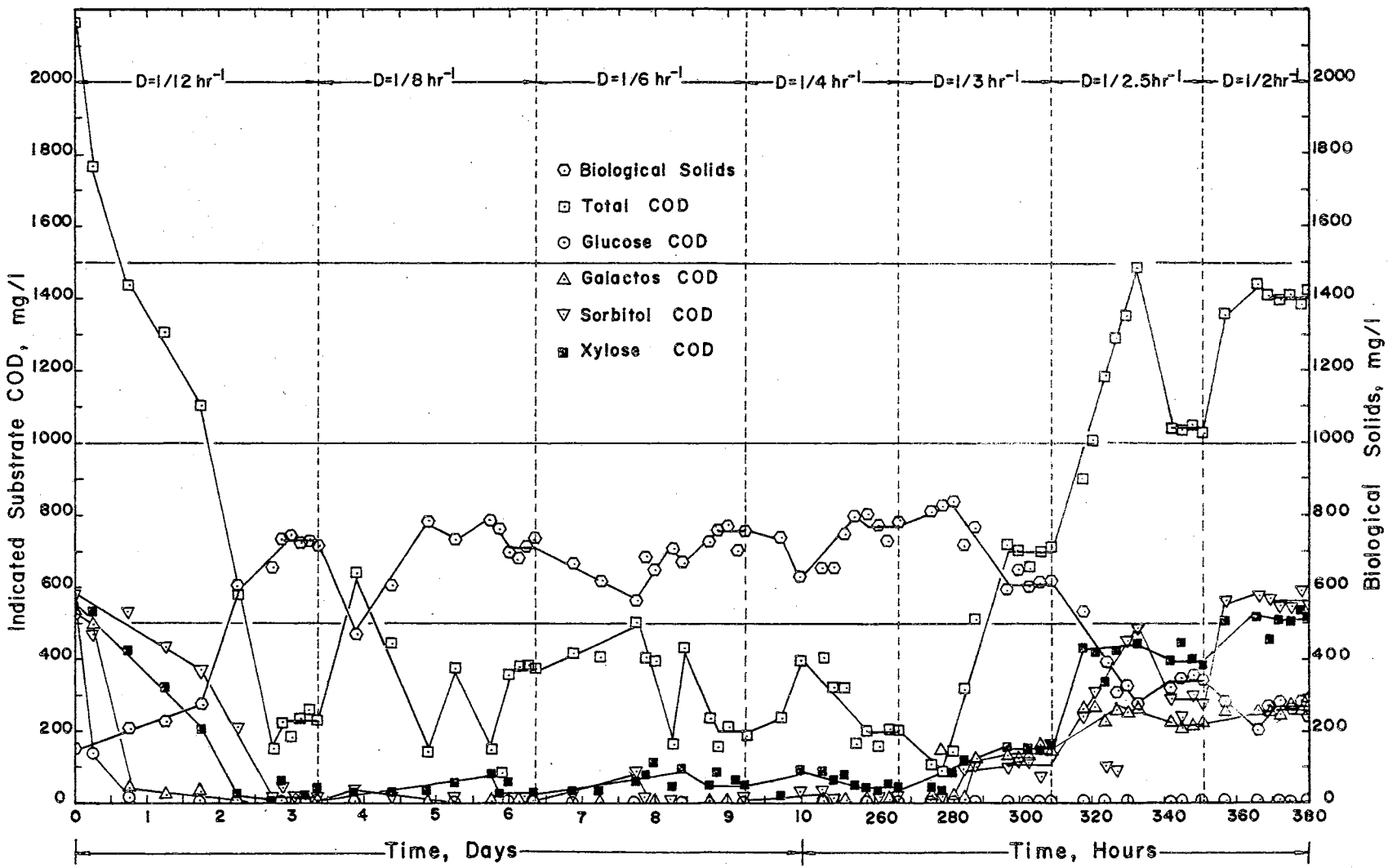

Figure 61 - System performance in the continuous flow activated sludge unit growing on glucose, galactose, xylose, and sorbitol at various dilution rates.

A change in dilution rate from 1/12 hr^{-1} to 1/8 hr^{-1} caused a rather severe transient disturbance. The effluent COD attained a peak value of 640 mg/l (at 3.9 days), and then decreased; eventually a new steady level (approximately 370 mg/l) was attained. The biological solids concentration dropped to a minimum value of 470 mg/l, and then built up to approximately 710 mg/l at the new steady-state. Only trace amounts of sorbitol and xylose were found in the effluent throughout operation at this dilution rate. At the new steady-state conditions, the cell yield was 40 per cent while the treatment efficiency was 83 per cent.

Considerable fluctuations in effluent COD and biological solids level were observed during the period of operation at dilution rates of 1/6 and 1/4 hr^{-1}. However, the degree of system disruption was rather small compared with that at D = 1/8 hr^{-1}. Throughout operation at these two dilution rates, only small amounts of xylose and sorbitol were detected in the effluent. It is interesting to note that the steady-state parameters for these two dilution rates were much the same (approximately; solids 760 mg/l, effluent COD 200 mg/l, xylose COD 50 mg/l, traces of sorbitol, and E = 90 per cent, Y = 39 per cent).

It is apparent from the figure that both biological solids and COD were being diluted out of the reactor to a considerable extent when the flow rate was increased to 1/3 hr^{-1}. After the transient was completed (about 1.25 days or 10 detention times), the biological solids level remained fairly constant at approximately 610 mg/l (Y = 42 per cent) while effluent COD rose to approximately 700 mg/l (E = 68 per cent). In the steady-state condition, the approximate leakages of substrates were: 150 mg/l xylose, 140 mg/l galactose, and 110 mg/l sorbitol.

When the dilution rate was increased to 1/2.5 hr^{-1}, a well-defined transient ensued. The efficiency of the system was disrupted severely. The biological solids concentration dropped to nearly 270 mg/l (at 13.9 days), and then rose slightly to a new steady-state value of approximately 340 mg/l (Y = 31 per cent). During this period, the total COD was diluted out to nearly 1480 mg/l, but rapidly returned to a new level of approximately 1040 mg/l (E = 52 per cent) in response to the recovery in solids concentration. A considerable portion of the effluent COD was attributable to leakage of the specific substrates in the feed. The peak transient substrate leakages were: sorbitol 480 mg/l, xylose 440 mg/l, and galactose 260 mg/l, whereas the steady-state values were: xylose 390 mg/l, sorbitol 290 mg/l, and galactose 220 mg/l.

The last hydraulic shock load applied to this system was a change in dilution rate from 1/2.5 hr^{-1} to 1/2 hr^{-1}. It should be noted that the shock brought about the development of large loosely-bound flocs in the mixed liquor which had a tendency to settle in the OD tube; therefore it was very difficult to adjudge the degree of complete mixing by measuring the OD of the reactor mixed liquor and the reactor effluent. Although the system finally exhibited a new "steady-state" behavior (within approximately 18 hrs or 9 detention times), there appears to be little doubt that the system was operating close to the maximum growth rate and severe cell dilute-out was occurring. The bulk of the "steady-state" effluent COD (approximately 1400 mg/l, E = 35 per cent) was attributable to sorbitol (560 mg/l), xylose (510 mg/l) and galactose (260 mg/l). The new solids concentration was approximately 270 mg/l, which provided a cell yield of 36 per cent.

Figure 62a shows the various steady-state parameters taken from

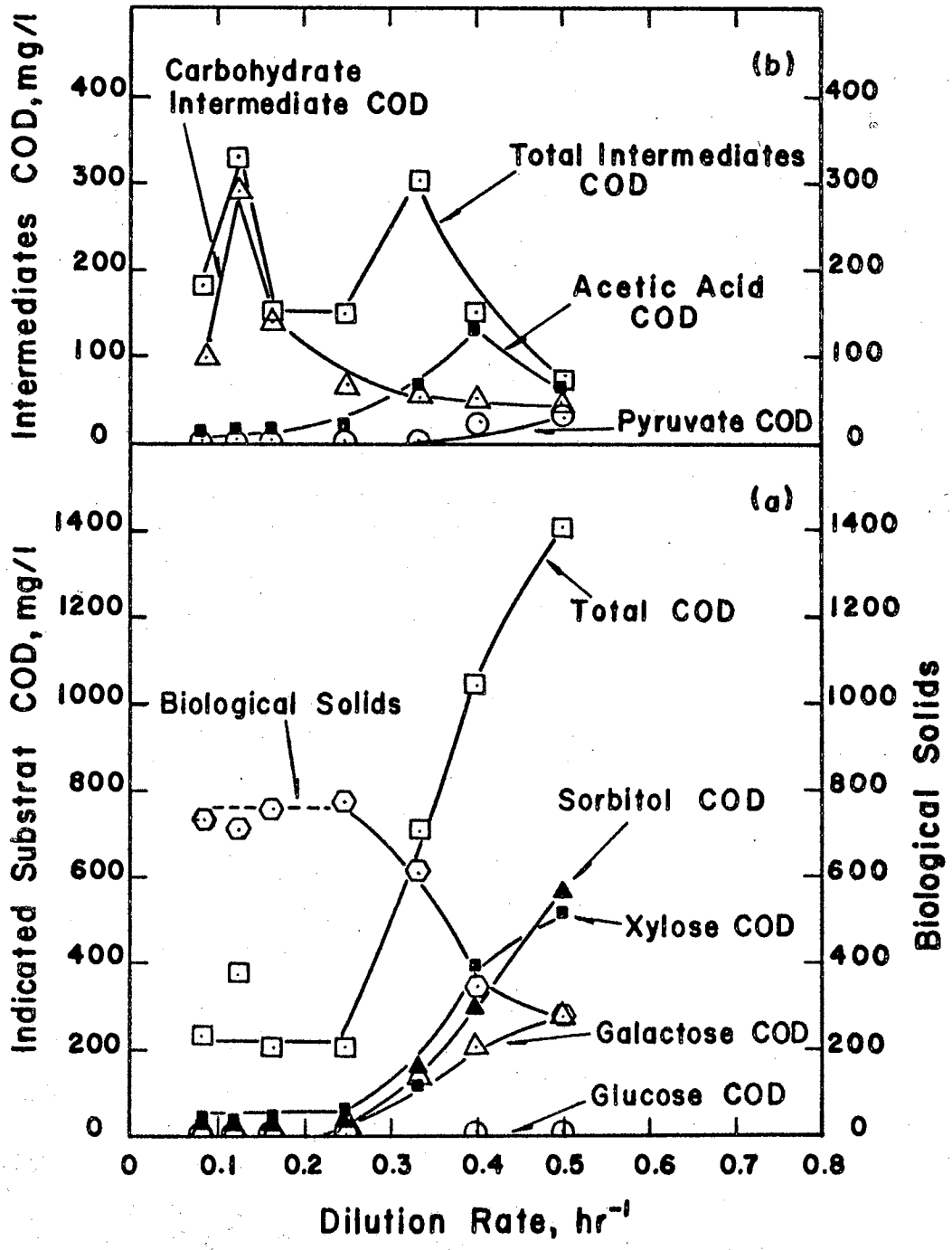

Figure 62 - (a) Metabolic responses in the steady-state continuous flow unit with a mixed feed of glucose, galactose, xylose, and sorbitol at various dilution rates; (b) intermediates accumulation.

Figure 61 for each dilution rate; Figure 62b shows the amounts of various metabolic intermediates accumulated at each dilution rate. In Figure 62a, it is seen that all of the carbon sources were completely consumed at dilution rates of 0.25 hr^{-1} or less. The approximately 40 mg/l of xylose COD at these dilution rates may have been due to orcinol-reactive metabolic intermediates produced during the catabolism of other substrates. Glucose was the preferred carbon source in this four-component substrate system and was completely removed throughout this series of experiments even at D = 0.5 hr^{-1}. It appeared that the system began to undergo dilute-out with respect to COD and solids when the reactor was operating at dilution rate exceeding 0.25 hr^{-1}. Xylose, sorbitol, and galactose began to appear in the effluent at 1/3 hr^{-1} (0.33 hr^{-1} dilution rate). It is interesting to note that substantial excretion of metabolic intermediates (300 mg/l) was also observed and that a considerable amount of acetic acid (80 mg/l) began to appear in the effluent at this period. As the dilution rate increased to 0.4 hr^{-1} the amount of substrate leakage rapidly increased and the acetic acid concentration attained a peak value of 130 mg/l, while trace amounts of pyruvate began to excrete into the reactor liquor. When the reactor was operating at the highest dilution rate of D = 0.5 hr^{-1}, the efficiency of glucose removal was still 100 per cent, but the efficiency of galactose removal dropped to 50 per cent and approximately 100 per cent of the xylose and sorbitol remained unutilized. At this dilution rate the intermediates were found to consist of approximately 50 mg/l each of acetic acid and carbohydrate and 30 mg/l pyruvate.

L. Long-term Batch Studies on Substrate and Solids Removal Under Growing and Nonproliferating Conditions During Metabolism of Glucose

The results of the short-term batch experiments described earlier in this report indicated that the largest conversion of original substrate into intermediary products was generally observed when glucose was the substrate. Also, previous research by Thabaraj and Gaudy (55) has shown that in systems run at the same loading levels as used herein, nonproliferating systems produced more extracellular products than did the growing systems, and that the endogenous oxidation of nitrogen-deficient sludge was much slower than that of protein-rich sludges. In the light of these findings, a systematic long-term batch experiment was undertaken to study the patterns and the extent of removal of the original substrate, intermediary extracellular products and endogenous cellular materials under both growing and nonproliferating conditions. One growing system (Figure 63) and three nonproliferating systems (Figures 64, 65, and 66) were examined during this study. The initial COD and biological solids ratio used in this stdy was 30:1, i.e., 3000 mg/l of glucose and about 100 mg/l initial biological solids. For convenience of presentation and comparison of results, the metabolic responses of the systems are divided into the following three phases:

a. Substrate Removal Phase (or Solids Accumulation Phase)

The substrate removal patterns were drastically different in the growing and the nonproliferating systems. It is seen that only 12 hours were required for the elimination of more than 3000 mg/l COD from the medium under growth conditions (Figure 63), whereas in the systems devoid of nitrogen, 235 hrs (Figures 65 and 66), or 400 hrs (Figure 64) were required. At the end of the substrate removal period, only a small amount of COD (less than 100 mg/l) remained in the growth system, but considerable residual COD (about 400 mg/l) was found in the

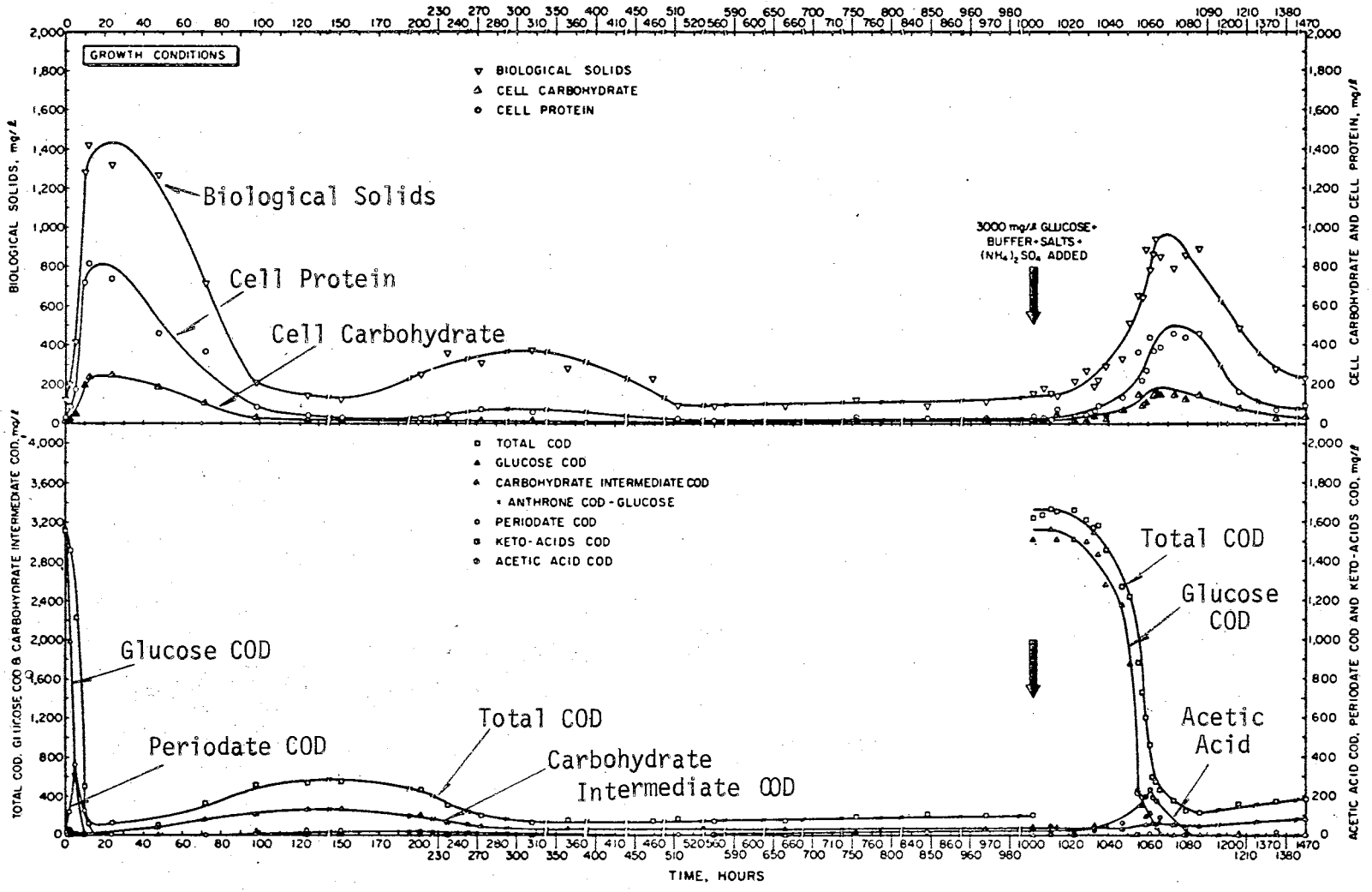

Figure 63 - Long-term batch system performance under growing conditions during metabolism of glucose (unit I).

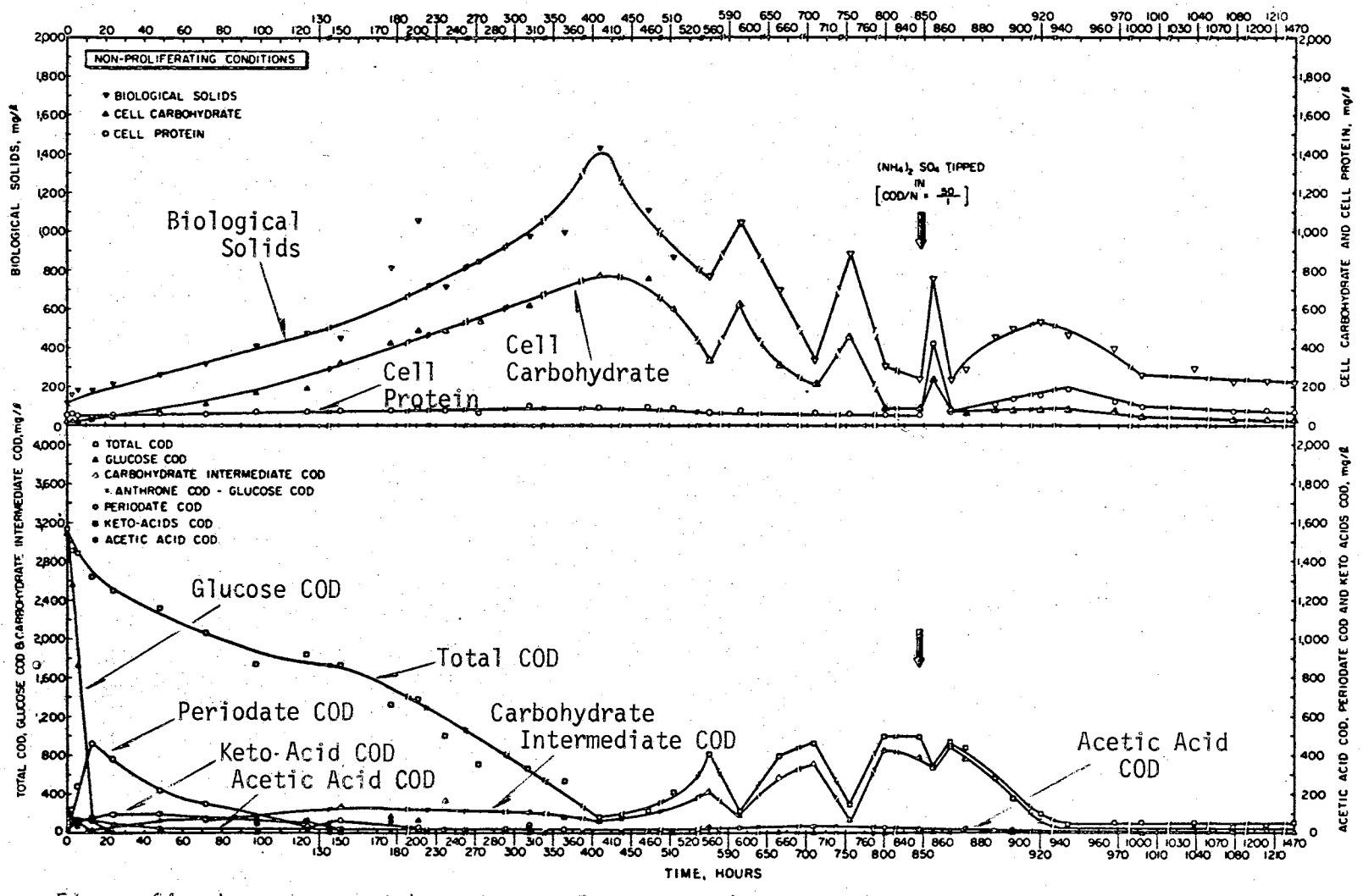

Figure 64 - Long-term batch system performance under nonproliferating conditions during metabolism of glucose (unit II).

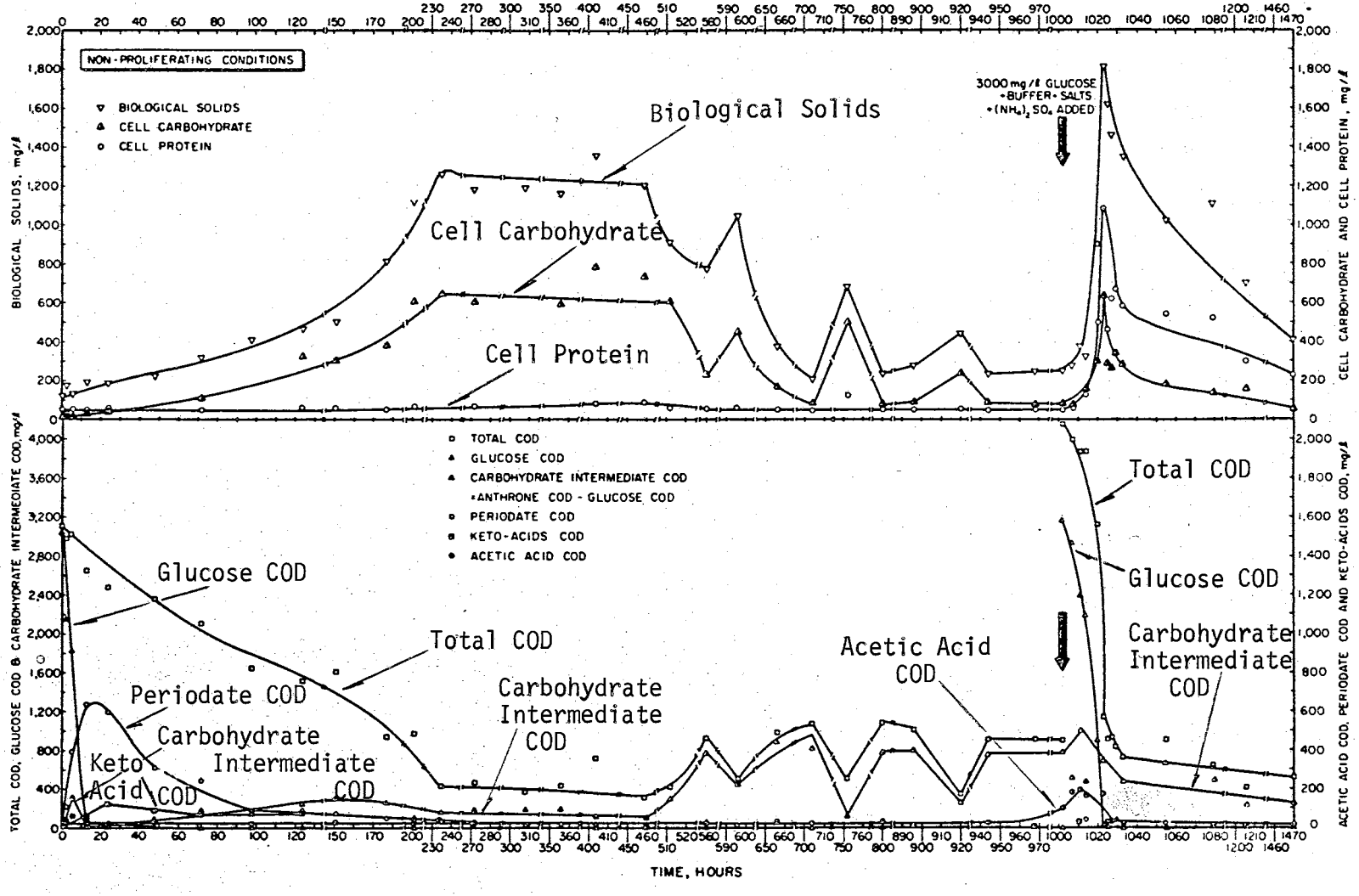

Figure 65 - Long-term batch system performance under nonproliferating conditions during metabolism of glucose (unit III).

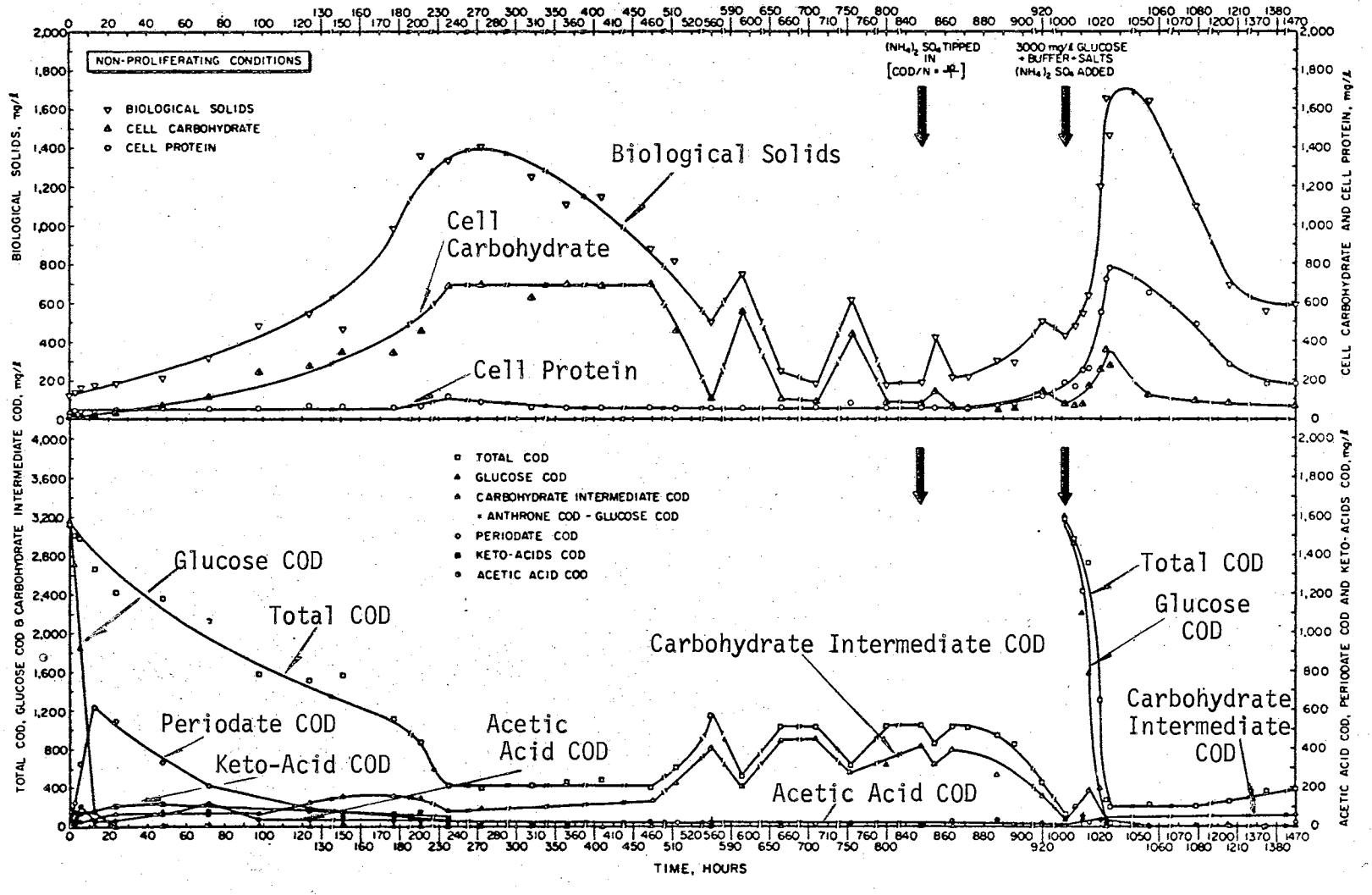

Figure 66 - Long-term batch system performance under nonproliferating conditions during metabolism of glucose (unit IV).

nitrogen-deficient systems. It is interesting to note that although the COD removal in the nitrogen-deficient systems was much slower than that in the growth system, the rate of elimination of glucose from the medium was comparable in the growing and nonproliferating systems. Glucose was removed in approximately 12 hours in the three nonproliferating systems. The pattern of COD removal in the growth system was essentially exponential (increasing first-order kinetics) during glucose metabolism, whereas two phases of decreasing first-order kinetics of different rates were observed in the nitrogen-deficient systems. This significant difference in system kinetics was also discerned from semilogarithmic plots of COD removal versus time (not shown).

The synthesis and accumulation of biological solids were also drastically different in these two systems. In correspondence with the kinetic difference in COD removal, the solids accumulation was exponential in the growing system, but the nonproliferating systems exhibited diphasic first-order reactions with different rates. There was some difference in the metabolic responses of the three nonproliferating systems even though the reactors were operated under the same conditions; however, the general trends were the same. The nature and amount of cellular materials synthesized in the growing and nonproliferating systems were significantly different. In the growth system, a larger portion of the consumed substrate was channeled into the synthesis of cellular protein, indicating growth and increase in microbial population. A small portion of solids accumulation was due to the synthesis of cellular carbohydrate. In the nitrogen-deficient system, more than 50 per cent of the increase in cell mass was due to the synthesis of carbohydrate and no significant synthesis of protein occurred.

At the times of peak solids concentration, the protein content of the cells in the growing system was 57 per cent, whereas 7 per cent (Figure 64), 5 per cent (Figure 65), and 6 per cent (Figure 66) of the nonproliferating cell dry weight was protein. On the other hand, carbohydrate constituted only 16 per cent of the accumulated biological solids in the growing system compared with 54 per cent (Figure 64), 49 per cent (Figure 65), and 50 per cent (Figure 66) for the nonproliferating systems. It is noted that the cell yields were comparable in the two systems (47 per cent for the growing system and Figures 64 and 65, 50 per cent for Figure 66).

The metabolic responses presented thus far indicate that nonproliferating cells produced considerably larger amounts of non-glucose COD than did the growing cells. Under nonproliferating conditions, nearly 85 per cent of the initial COD was convered into metabolic intermediates and/or endproducts during the first 12 hours of metabolism, whereas only about 5 per cent was observed in the growing system; these intermediates were subsequently utilized.

During the substrate removal (or solids accumulation) phase, traces of acetic acid (approximately 20-30 mg/l) and a significant amount of keto acids (approximately 200 mg/l) were present in the nonproliferating systems. In the growing system, a considerable accumulation of periodate-reactive intermediates (about 700 mg/l) occurred before the exhaustion of glucose. The nonproliferating cells released periodate-reactive materials to a greater extent than did the growing cells (e.g., the peak concentrations were 920 mg/l, 1230 mg/l, and 1210 mg/l for Figures 64, 65, and 66, respectively.) It was reasoned that these periodate-reactive materials were probably not sugar alcohols

such as sorbitol, glycerol, etc., since the system was highly aerobic and such reduction products of glucose metabolism would not be expected in an aerobic system. In such a system it would be expected that acid intermediates could be elaborated. In some systems, both acetic and pyruvic acids were found to be present but these compounds did not react with periodate. It is known that some organisms can cause gluconic acid to accumulate in the medium (56). There was no readily-available analysis for gluconic acid, but it was determined experimentally that gluconic acid did give a significant periodate reaction. It was found that over a wide range of concentrations (0.08 to 0.50 mg) gluconic acid yielded a color reaction equivalent, approximately, to one-third that for the same amount by weight of the glycerol standards. Small amounts of non-glucose carbohydrate (anthrone) were also present in the nitrogen-deficient systems.

b. Solids Removal (or Endogenous) Phase

From the figures it can be seen that endogenous oxidation of solids was also drastically different in the growing and nonproliferating systems. Under growing conditions, almost "total oxidation" of the accumulated solids was observed at 120 hours. Within approximately 110 hours of endogenous metabolism, the biological solids dropped from the peak accumulation (1400 mg/l) to the initial solids level (about 120 mg/l). Corresponding to the oxidation of cells, there was some release of cellular COD (about 50 per cent was attributable to carbohydrate). The release began at the onset of the endogenous phase and reached its maximum level at approximately 560 mg/l (about 260 mg/l was attributable to carbohydrate) at the time of attainment of "total oxidation." Although cellular protein and cellular carbohydrate were

removed concurrently, the oxidation of protein was more rapid than that of carbohydrate. During the "total oxidation" period, the levels of both cellular carbohydrate and protein returned to their initial values. There was a considerable lag (from approximately 120 hr to 150 hr) in this system after the solids oxidation phase, and then the remaining cells grew on the intermediary COD which had been released into the medium during the solids oxidation phase. It took approximately 160 hours (from 150 to 310 hours) to remove the remaining COD and to attain the previous low residual COD level (about 100 mg/l). At this time, the corresponding solids concentration was approximately 360 mg/l. Upon the exhaustion of filtrate COD, another period of solids oxidation started. The process of solids oxidation in this period was much slower than that in the first phase. Approximately 200 hours (310-510 hours) was required to complete the "total oxidation" of 260 mg/l solids. During this endogenous metabolism period, no liberation of soluble COD was observed. Thus at 510 hours, the system attained the optimum treatment with respect to both substrate and solids, and appeared to attain a "resting" status.

The endogenous metabolism phases of the three nonproliferating systems were not exactly alike; however, in general they were comparable. Generally, the endogenous oxidation of nonproliferating cells was very slow. It is seen that solids degradation occurred after the attainment of the peak level in Figures 64 and 66, but a prolonged stationary phase was observed for the system shown in Figure 65. It is interesting to note that after a long period of slow and steady oxidation, a considerable fluctuation in the concentrations of biological solids and corresponding filtrate COD occurred and there was a gradual decrease in the

levels of these two parameters. Cell protein remained stable in these units during the endogenous phase, whereas cellular carbohydrate fluctuated up and down corresponding to the fluctuations in biological solids concentration. A large portion (more than half) of the filtrate COD was attributable to carbohydrate. Only traces of acetic acid were present throughout the endogenous phase.

In order to study the metabolic responses of the nonproliferating cells to the addition of nitrogen source, ammonium sulfate $(NH_4)_2$ was added to the systems to yield a COD/N ratio of 10:1 in Figure 66, and COD/N of 50:1 in Figure 64. A rapid synthesis of biological solids occurred upon the addition of nitrogen source, and was followed by a rapid return to the level which existed previously under nonproliferating conditions. Thereafter, a slow buildup of microbial population was accompanied by a gradual removal of filtrate COD. It is seen that the addition of nitrogen source initiated the synthesis of cellular protein and enhanced the removal of filtrate COD: the cellular protein concentration was higher than that of cellular carbohydrate during this period. At approximately 920 hrs (Figure 64), filtrate COD had been completely utilized and more than 500 mg/l of solids were present in the reactor. The solids level was then gradually reduced; however, about 220 mg/l of solids was found at the end of this experiment (1470 hours). It is noted that throughout the endogenous phase between 50 and 100 per cent of the released metabolic intermediates were carbohydrates. It seems that the amount of nitrogen source added to the nonproliferating systems did not cause a significant difference in metabolic responses as shown by Figures 64 and 66. A possible explanation for the similarity of results is that the ratio of COD/N was based on

the initial substrate concentration (3000 mg/l) not on the remaining COD (approximately 500 mg/l) and therefore both nitrogen feedings (COD/N = 50:1 and 10:1) provided excess nitrogen supply for the cells to metabolize the remaining COD under balanced growing conditions.

c. Refeeding Phase

In order to examine the substrate removal capability of the organisms after the long-term endogenous operation, 3000 mg/l of glucose and the required nutritional elements were added to the reactors of Figures 63, 65, and 66 at 1002 hours. It is seen that both nonproliferating systems in Figure 65 and the newly-formed growing system in Figure 66 were capable of removing glucose without any lag period. In these two units, the cells consumed all of the glucose within 20 hours, and biological solids concentration increased to 1800 mg/l (Figure 65) and 1700 mg/l (Figure 66). Since both units were under growing conditions, the cells synthesized more cellular protein than cellular carbohydrate. Both cell protein and carbohydrate peaked at the time of maximum solids accumulation, and constituted 61 and 22 per cent, respectively, of the cell dry weight in Figure 65, and 45 and 20 per cent, respectively, in Figure 66. Upon substrate exhaustion, the organisms initiated endogenous metabolism and oxidized the accumulated solids. At the end of the experiments (1470 hours), 400 mg/l and 600 mg/l of biological solids were present in Figures 65 and 66, respectively. Although endogenous oxidation of biological solids in these two reactors was not completed at the termination of the experiment by this time, more than 85 per cent (approximately 88 per cent for Figure 65 and 85 per cent for Figure 66) of the net solids synthesized during the solids accumulation phase had been oxidized. It is interesting to note that metabolic

products were not accumulated in the medium during endogenous oxidation of the solids.

From Figure 63 it is seen that the long-term endogenous period caused a lag in the growth response upon the introduction of glucose and other nutrients. After a lag period of 10-15 hours, the organisms began to remove the substrate and approximately 50 hours were required to consume 3000 mg/l of glucose. This removal rate was rather slow as compared to the 12 hours which was required for the first-round feeding of 3000 mg/l glucose and the 20 hours in Figures 65 and 66 for the second-round feeding of glucose. The maximum solids concentration was 970 mg/l, which indicated very low cell yield (approximately 30 per cent). The approximate cell yield in the first-round feeding was 47 per cent, and yields were 45 and 42 per cent, respectively, for the second-round feedings of Figures 65 and 66. A considerable amount of acetic acid was released during metabolism of glucose. Liberation of acetic acid attained its peak (approximately 200 mg/l) at the time of glucose exhaustion. Thereafter, the acetic acid was consumed. At the termination of this experiment, only 200 mg/l of solids remained in the reactor; however, about 400 mg/l of residual COD persisted in the medium. It is significant to note that the biological solids which had undergone a very long resting or endogenous period before refeeding did exhibit a satisfactory growth response (although there was a lag). Therefore, one cannot consider these solids to have been biologically "inert."

CHAPTER V

DISCUSSION

A. Substrate Interactions in Multicomponent Carbon Source Systems

Previous studies in the Oklahoma State University bioenvironmental laboratories have shown the existence of competitive and repressive interactions between various carbon sources in both batch and continuous flow systems. The present work was designed to verify and extend the past effort. Due to the extensive amount of experimental data obtained in the present experimental effort as well as the great amount of data obtained previously, an attempt is made in this section to summarize the previous findings in relation to the present findings and to bring the significant trends of the responses into focus.

The results of the studies herein reported seem to demonstrate that substrate interactions resulting from the operation of metabolic control mechanisms are manifest during the removal of various three-component and four-component carbon source systems employing heterogeneous microbial populations in either batch or continuous flow experiments. Furthermore, the present findings provide the most massive amount of evidence for the ubiquity of control mechanisms in natural populations under continuous culture conditions. At this point it should be emphasized that several possible alternative control mechanisms can be employed in the interpretation of any pattern of substrate removal which

was observed, i.e., one cannot, from these results, determine with finality which of the various control mechanisms were operative. It is also important to understand that observation of concurrent removal is not unequivocal evidence against the operation of control mechanisms (repression and/or inhibition), because repression can be expressed as a decrease in the rate of enzyme synthesis (not complete cessation of synthesis) and inhibition can retard the rate as well as totally stop enzyme activity. Therefore, as stated in the previous chapter, the idea of "reference time" was introduced to help assess the degree of substrate interference which occurred in the presence of other carbon sources.

The fact that leakage of some substrates (such as glycerol, sorbitol, xylose, propionic acid, and butyric acid in Figures 18, 34, 42, 50, 52, and 62, respectively) gradually increased with increase in dilution rate indicates in some measure that higher growth rate (or younger cell age) fosters greater manifestation of substrate interference by either repression or inhibition. In other words, when the cell age decreased (or dilution rate increased) the system was subjected to more severe substrate interference. The results obtained from the batch experiments (zero dilution rate) also show the tendency for manifestation of substrate blockage. The finding that the pattern of substrate removal shifts toward a more sequential mode as the growth rate increases or as the "age" decreases also has been reported for past studies in the bioenvironmental engineering laboratories of Oklahoma State University (4)(14)(15)(57).

The data for single-substrate batch systems show that in general the growth and/or accumulation of intermediates, when glucose was the

sole carbon source, was significantly greater than for the other sole carbon sources. Thus glucose was the most readily available carbon source and possessed higher potential for interference with the utilization of other substrates. The results for the combined substrate batch systems show that the utilization of other carbon sources was partially (e.g., sucrose in Figure 32) or completely (e.g., ribose in Figure 16) blocked by the metabolism of glucose. These results clearly show that criteria such as growth rate, accumulation of metabolic intermediates and acclimation period (if required to initiate metabolism) in the single substrate systems can be used to estimate the relative potential a substrate may possess for interference with the utilization of another substrate when they are present in a mixture. However, based upon the present results, it is felt that the relative growth rates (more than intermediate accumulation) provide a better basis for judgment of relative interference potential between two compounds (when no acclimation period is required for either compound). For example, in Figure 55, the glucose control exhibited a slightly higher growth rate but slightly smaller accumulation of intermediates than did the xylose control, and the relative inhibitory potential of these two compounds was predicted to be glucose > xylose. In the combined system, xylose removal was totally blocked until glucose was removed (Figure 56).

There are three primary reasons for adjudging growth rate to be a more credible indicator for assessing relative interference potential. Firstly, the accumulation of organic metabolites in the medium during growth on compound "A" may not represent an ability for rapid metabolism of the carbon source and a tendency to build up temporary products which feed back to control metabolism of "A" or other carbon sources.

Accumulation of product may simply represent formation of an endproduct of metabolism of that carbon source. Secondly, the observations made can only assess the rate of accumulation of intermediates and/or endproducts, not their rate of production. The system exhibiting slight accumulation of products may be producing them (and using them) much more rapidly than the system exhibiting the greater accumulation. Thirdly, it cannot be said unequivocally whether catabolites or other factors govern the fine scale regulation of enzyme activity. This question will be discussed in the next section.

It is interesting to note that in Figure 13, sorbitol was metabolized slightly more rapidly than was glucose, and that the metabolism of sorbitol produced a slightly higher accumulation of intermediates than did that of glucose. According to criteria employed previously for predicting relative interference potential, sorbitol should possess relatively higher potential to interfere with glucose utilization. On the contrary, sorbitol removal was inhibited by glucose when these two compounds and ribose were used as combined substrate (Figure 14). At this point, it should be emphasized that estimation of the relative potential for substrate interference (based upon growth rate and accumulation of organic metabolic products) provides only a rough guide for estimating the effect of the presence of one carbon source on the removal of another.

The secondary substrate, e.g., sucrose in the glucose-sucrose-xylose system (Figures 29, 30), glycerol in the glucose-glycerol-butyric acid system (Figures 51, 52), also interfered with the utilization of the least available substrate (xylose and butyric acid in these two systems). Therefore it may be concluded that substrate interference is not an effect uniquely caused by glucose and that the utilization of a

third substrate (the least available substrate as adjudged by the control) may be subject to interference by both the first substrate (glucose in the present case) and the secondary substrate. However, in some cases the interference between secondary and tertiary substrates is not so evident and definite as that between first and secondary, and between first and tertiary substrates. For example, the presence of glycerol seemed to retard slightly the utilization of ribose in the glucose-acclimated cell system (Figure 36) and ribose-acclimated cell system (Figure 38), whereas glycerol removal was retarded by ribose in the glycerol-acclimated cell system or ribose removal was enhanced by the presence of glycerol (Figure 40). One possible explanation for the different results in glycerol-acclimated cell systems is that the glycerol COD curve determined by the periodate test did not necessarily represent the actual glycerol concentration because the metabolism of glucose and ribose also may produce periodate-reactive intermediates. Heidman (4) found that sorbitol-acclimated cells produced considerable amounts of periodate-reactive intermediates during the metabolism of glucose. Therefore, the glycerol COD curve may represent the sum of glycerol and periodate-reactive products from glucose and ribose. It is felt that a new or modified analytical technique, which is specific only for sugar alcohol, is needed for the study on substrate interactions between sugar alcohols and other carbon sources. The test employed is the best one available. Labeled sorbitol could be employed, but its use would be excessively expensive for any long-term study. Heidman (4) attempted to arrive at an estimate of actual sorbitol concentration by subtracting the periodate-reactive intermediates in a glucose control from "sorbitol COD" in the combined system. While this

was a worthwhile approach, it does not solve the problem. As has been pointed out previously (see Figures 3 and 4, 5 and 6), the amount of intermediates accumulating from glucose metabolism in the control system and in the combined system are not necessarily the same. Furthermore, it is possible that the metabolism of the sugar alcohol itself and of the third substrate may also produce periodate-reactive intermediates.

Another possible explanation for the lesser interaction between the secondary and tertiary substrates is that the difference in interference potential between these two substrates is much smaller than that between glucose (first substrate) and either of these substrates. The pattern of interaction between the second and third carbon sources may be more easily subject to influences of environmental changes, e.g., intermediate production, pH, dissolved oxygen, and changes in microbial species in the population. In this regard, attention is called to the results for the glucose-galactose-fructose system. Based on growth rate and accumulation of intermediates in the controls (Figures 3, 5, and 7), it can be seen that the relative interference potentials of galactose and fructose were similar and much smaller than that of glucose with the exception of the fructose-acclimated cells (Figure 7). When these three sugars were used as the combined substrate, the sequence of removal was glucose > fructose > galactose in the systems using galactose-acclimated cells (Figure 6) and fructose-acclimated cells (Figure 8), but was glucose > galactose > fructose for glucose-acclimated cells (Figure 4). Although the sequences of removal of these three sugars were not the same in all three systems, all of them were consistent with the relative potential sequences predictable from their behavior in the appropriate controls. This suggests that in general the potential

sequence observed in the controls can be used for predicting the order of substrate removal in the combined systems. It is interesting to note that the metabolism of galactose by glucose-acclimated cells (Figure 3) produced more intermediates than did its metabolism by other sludges (Figures 5 and 7) and that a considerable amount of intermediates (more than 450 mg/l) was accumulated at the time when galactose was exhausted in the combined system by glucose-acclimated cells (Figure 4). These observations provide some indication that the shift of priority from fructose > galactose to galactose > fructose might be attributable to the intermediates accumulated during galactose metabolism. In addition to the glucose-galactose-fructose system, the substrate removal sequence exhibited in glucose-galactose-sucrose, glucose-sorbitol-ribose and glucose-glycerol-ribose systems was affected by the "acclimation history" of the population employed. However, the removal sequences for the other three batch systems were glucose > sucrose > xylose, glucose > sucrose > glycerol, and glucose > galactose > xylose > sorbitol, regardless of the acclimation history of the population.

It is interesting to note that the results for the continuous flow systems conducted in this study clearly indicate that the order of priority for substrate removal was consistent throughout the experiment regardless of various changes in microbial predominance which resulted from shifting the dilution rates. The sequences for these multicomponent systems were: glucose > galactose > fructose; glucose > sucrose > galactose; glucose > sucrose > xylose; glucose > galactose > sorbitol > xylose; glucose > sorbitol > xylose; glucose > glycerol > xylose; glucose > glycerol > butyric acid and glucose > sorbitol > propionic acid.

B. Excretion of Intermediary Metabolites and Control Mechanisms

With most of the systems studied, significant amounts of metabolic intermediates and/or endproducts were accumulated in both batch and continuous flow mixed substrate media. The results show that, generally, metabolism of glucose led to the largest accumulation of metabolic intermediates. Other substrates which occasionally gave rise to large amounts of intermediary metabolites were fructose, sucrose, and galactose, which were metabolized at slower rates than glucose, but at greater rates than were compounds such as ribose, propionic acid, etc. These excreted products may have been metabolized later by the cells which produced them or by other organisms, but it is not possible to determine which segments of the population utilized them from the results of these experiments.

No attempt was made to analyze the nature of the metabolic intermediates accumulated in the batch systems. In most cases studied, the peak concentration of metabolic products in the combined system was accompanied by the exhaustion of the preferred substrate (glucose). These results imply that the intermediates excreted from glucose metabolism may have played an important role in development of interference with utilization of other substrates present in the systems.

In the continuous flow systems, significant amounts of metabolites were found in systems fed with such combinations of carbon sources as glucose-fructose-galactose, glucose-galactose-sucrose, glucose-sucrose-xylose, glucose-sorbitol-propionic acid, glucose-glycerol-butyric acid, and glucose-galactose-xylose-sorbitol (see Figures 10, 26, 34, 50, and 62). In these cases, excretion of organic metabolites was accompanied, usually, by the presence in the effluent of one or more of the substrates

fed to the microorganisms. The results shown in these figures indicate two distinct trends. First, the concentration of accumulated catabolites in the effluent rises gradually with increasing dilution rate and with a concomitant sharp rise in the concentration of the "less-preferred" feed substrates (Figures 10, 52). Secondly, the concentrations of both the accumulated intermediates and the less-preferred substrates in the effluent rise sharply within a very narrow range of dilution rates followed by a gradual decrease in the concentration of excreted catabolites, a continuing rise in concentration of the less-preferred feed substrates, and finally, leakage of glucose (Figures 26, 50, and 62).

The analyses made for identification of accumulated intermediates indicate that considerable excretion of acetate occurred at moderate and high dilution rates in the systems containing glucose-fructose-galactose (Figure 10), glucose-galactose-sucrose (Figure 26), glucose-sorbitol-propionic acid (Figure 50), and glucose-galactose-xylose-sorbitol (Figure 62). The two distinct trends noted for accumulation of all intermediates were also observed for the accumulation of acetate specifically. Generally the excretion of pyruvate was not significant. Small amounts of pyruvate were observed in the experiments shown in Figures 10 (50 mg/l), 18 (20 mg/l), and 26 (90 mg/l). In addition to the acetate, pyruvate, and anthrone-reactive carbohydrates, other unidentified metabolites also accumulated in the medium (as indicated by the difference between total intermediate COD and the sum of the COD's of the identified metabolites).

It is emphasized that the products which accumulated in the medium cannot be construed as possible fermentation products due to oxygen

limitation. Aerobic conditions were carefully maintained, and periodic checks on dissolved oxygen concentrations in both batch and continuous flow systems revealed levels between 3 and 6 mg/l. There are many reports concerning the excretion of metabolic intermediates and/or end-products from the metabolism of substrates such as glucose, sucrose, etc., in cultures grown aerobically. In 1956, Neidhardt and Magasanik (58) observed that an extremely large amount of glucose was metabolized by Aerobacter aerogenes under aerobic conditions. From these results they concluded that large amounts of intermediate products must be either accumulated in the cells or excreted into the medium. Later, in 1963, Clifton (59) found that a large amount (50 per cent of glucose carbon) of both volatile and nonvolatile organic acids accumulated in the medium during aerobic growth of Escherichia coli K-12 on glucose. Bhatla and Gaudy (60) investigated twenty-one pure cultures and found that one species of Escherichia excreted acetate during aerobic growth of cells on glucose. Eagon and Cho (61) reported that Pseudomonas natriegens also produced acidic products, such as acetate, pyruvate, and lactate, when grown in carbohydrate broth under aerobic conditions. There is also some evidence for production of acetate and other catabolites in continuous flow experiments, e.g., with Aerobacter species grown on glucose (62) and with Neisseria gonorrhoeae grown on glucose-peptone (63).

Some information concerning the excretion of acetate or other intermediates by heterogeneous microbial populations is also available. Adamse (64) observed the release of acid intermediates, mainly acetic acid, during the metabolism of lactose (dairy wastes). Krishnan and Gaudy have observed that considerable amounts of metabolic intermediates

and/or endproducts are released during glucose metabolism by glycerol-acclimated cells under either growing or nonproliferating conditions (65). Recently, Thabaraj (66) observed that during metabolism of glucose, sucrose, glycerol, and sorbitol, acetate was an extracellular product, whereas pyruvate was identified in the glucose system only. A study on the metabolism of a multicomponent substrate medium during continuous culture of a mixed microorganisms was conducted by Chain and Mateles (25). They reported that a significant amount, as large as 10 to 20 per cent of the carbon source consumed, was excreted as acetate in glucose-butyrate and glucose-lactose systems under carbon-limiting conditions.

The accumulation of acetate in the medium by cells which are rapidly growing on glucose (Figures 10, 26, 50, and 62) may reflect a high ATP level in the cells. On the assumption that glucose metabolism is conducted through the Embden-Meyerhoff-Parnas (EMP) pathway, under aerobic conditions there is opportunity to produce a considerable amount of ATP in converting the carbon source to acetyl-CoA. It is known that ATP can inhibit citrate synthetase, the enzyme required for further oxidation of acetyl-CoA via the TCA cycle. The buildup of acetyl-CoA can be relieved through a reaction mediated by phosphotransferase, yielding acetyl phosphate. The acetyl phosphate can then be converted to acetate and one mole of ATP in a reaction controlled by acetate kinase. The latter reaction produces 1 ATP, as compared to 15 ATP's which would be derived from acetyl-CoA if it went through the Krebs cycle. Therefore, if a large quantity of ATP builds up with the EMP pathway, the cells can prevent further buildup by blocking the TCA cycle, thus controlling their energy metabolism. As a consequence, acetate

concentration would build up in the medium (67)(68)(69).

Metabolite inhibition in heterogeneous microbial systems has been demonstrated in some of the experiments in this study (e.g., Figures 4, 6, 8, 48, and 50) and by work of others [Gaudy and co-workers (8)(20), Prakasam and Dondero (16)(17), Stumm-Zollinger (18)(19), and Chain and Mateles (25)(26)]. This mechanism has only recently been recognized in the basic microbiological field (28)(13). Zwaig and Lin have shown that glycerol kinase is inhibited by fructose -1, 6-diphosphate, a metabolite which is formed during the metabolism of both glycerol and glucose. It seems probable that many metabolites common to catabolic pathways can inhibit activity of various enzymes. Much more research is needed to delineate the mechanisms further and to define various metabolites or other factors which may govern the action of these mechanisms. As pointed out previously, from the standpoint of understanding and perhaps eventually controlling the course of purification of polluted water in natural ecosystems, it is of more immediate importance to the bioenvironmental engineer to study the substrate interactions (i.e., the end result of the operation of the control mechanisms) than to study the basic control mechanisms themselves.

It has been pointed out in Chapter II that three possible types of sequential substrate removal can occur in heterogeneous populations. They occur: 1) between feeding substrates when two or more exogenous substrates are added (7); 2) between feeding substrate and intermediates when the cells produce exogenous metabolites during metabolism of an original external carbon source (31); and 3) between feeding substrate and cellular material when cellular materials are released as a result of a severe osmotic shock (32). The results of mixed-substrate studies

(short-term batch and continuous flow systems) provide many evidences for sequential removal of the types 1 (e.g., Figures 16 and 46) and 2 (e.g., Figures 22 and 60). Also it would seem that considerable amounts of metabolic intermediates were accumulated in the reactors during the period of glucose metabolism in the long-term batch studies. The peak concentration of intermediate product accumulation occurred at the time of exhaustion of glucose and the cells removed these intermediates as soon as the external glucose was consumed. Thus the mode of removal in both the growing and nonproliferating systems was essentially sequential removal (type 2). There was apparently little or no acclimation period required after glucose removal before initiation of metabolism of the intermediate products. It should also be noted that the kinetic mode of endogenous destruction of the biological solids differed in each system and there were (especially in Figures 64, 65, and 66) kinetic discontinuities suggestive of sequential degradation of portions of the population and/or different components of the cell. During the period of endodigestion, most of the released COD was accounted for as carbohydrate.

C. Microbiological Interaction in Heterogeneous Microorganisms

Since distinct changes in predominating species were frequently observed in this study, especially in continuous flow experiments, it is felt that some discussion concerning microbiological interactions may shed some light on the present research. Although both batch and continuous flow experiments were conducted in this investigation, primary attention will be given to the results of the continuous flow studies, since under such conditions environmental variables may be

expected to stay within relatively narrow ranges. Batch systems, on the other hand, undergo significant changes in substrate concentration, accumulation of intermediates, and possibly significant changes in pH, dissolved oxygen, and other key parameters, during an experiment.

In a batch system, when substrate is in abundance, the cells are growing near their maximum growth rate. The cells growing faster and having the higher μ_m eventually will predominate in the population. These cells may then die off when substrate becomes limiting, thus giving rise to a succession of predominating species. On the other hand, in a continuous flow system, where the limiting substrate (glucose) concentration is nearly zero at all times, the cells which have a higher affinity ("capture and retention") for the limiting substrate ultimately will predominate. Subsequently, the cells having lower affinity for the limiting substrate will tend to be washed out. Thus the population fluctuations occurring in continuous flow systems can be expected to be different from the "ecological succession" (70) observed in batch systems in which new species gain predominance as others produce nutrients or die and release cell substances.

At high dilution rates (where glucose appeared in the effluent, e.g., Figures 33 and 49), the systems generally became relatively unstable at or near the complete washout region. Heavy wall growth was often observed at high dilution rates and made sampling too difficult for accurate analyses to be performed. At this point, it would be well to comment on the reports of Edwards (42) and Bungay (39)(41). They observed that at very high dilution rates, the predominant species were surface-adhering organisms which resisted washing out. The results of the present study also indicated a heavy "wall growth" as described

previously. Furthermore, visual observation of the reactor effluent and mixed liquor revealed the presence of flocculated cells at high dilution rate.

Another interesting finding is that the maximum specific growth rate on a multicomponent substrate estimated from the batch systems is generally smaller than that observed in continuous flow systems. Since substrate concentrations as high as 1500 mg/l or 2000 mg/l (500 mg/l was glucose) were fed to the batch units, the growth rate obtained from the combined substrate system might be very close to the maximum growth rate, μ_m. It is seen from the Appendix (page 196) that very few of these values are higher than $\mu = 0.35\ hr^{-1}$. However, most of the continuous flow systems (except the glucose-galactose-sucrose system, Figure 26) were able to grow on the multicomponent substrate at a dilution rate (or growth rate) of $D = 0.5\ hr^{-1}$ without leakage of glucose. It is quite possible that an entirely different set of predominating organisms was involved in each of the systems (batch and continuous).

Generally it is thought that for a continuous (pure) culture grown on a single organic compound, a period of six detention times (or generations) of transient growth is adequate to attain a new "steady-state" provided spontaneous mutation rate is low (40). However, in a continuous flow system with heterogeneous population and multicomponent substrate, microbiological interactions should be considered in addition to the substrate interactions. The results of the present investigation suggest that in general at least ten detention times should be allowed at any dilution rate before the system is adjudged to be in steady-state. A transient period as long as twenty-three detention

times was found in the glucose-sorbitol-propionic acid system (Figure 49) at a 1/2 hr^{-1} dilution rate. Furthermore, due to substrate interactions and microbiological interactions which are exhibited in continuous flow systems, the data on effluent COD, biological solids level, and concentrations of specific substrates appear to fluctuate about some mean value, even under the so-called "steady-state" conditions. The relatively unstable "steady-state" which was attained much more slowly than in continuous pure culture on single carbon sources has also been observed in continuous flow studies with mixed known cultures (39)(40). It is conceivable that the fluctuations might be due to sampling or analytical errors and that they also might reflect real changes within the system. However, Gaudy, et al. (71) have observed that the kinetic constants varied for each dilution rate, and could not be considered as precise constants in depicting the kinetic behavior of heterogeneous populations. Thus the fluctuations most likely indicate real changes within the system. Because natural fluctuations might occur throughout the experiment, the terms "steady-state" or "equilibrium," when applied to continuous cultures of heterogeneous microbial populations, should be used to define an average condition. In other words, in applying "chemostat" studies to the design of an activated sludge system, one should base values on averages taken over extended periods of time rather than on one or two values obtained from a "steady-state" assumption. It should be noted that population changeover also may occur during extended periods of "steady-state" operation; for example, the 46-hour transient growth described above might have consisted of one or two "steady-state" periods separated by a population changeover. Therefore, caution (and patience) are needed in

defining new "steady-state" conditions and there is no substitute for taking sufficient numbers of samples. In this study, the changes in microbial population were adjudged by appearance and color of the mixed liquor, flocculation and settling characteristics of the sludge, and occasionally by microscopic observation. Population changeovers in the system, whether of one species replacing another or selection of mutants within a species, can occur without such obvious indications of changes. However, because these gross changes in predominance are evidenced, lesser, not easily detected, changes must be assumed to be taking place, probably even more frequently.

CHAPTER VI

CONCLUSION

1. Manifestations of the operation of metabolic control mechanisms during the removal of substrates in various three-component and four-component carbon source systems by heterogeneous microbial populations in either batch or continuous flow operation were observed to occur.

2. Higher growth rate (or younger cell age) fosters greater manifestation of substrate interference by either repression or inhibition.

3. Criteria such as growth rate, accumulation of metabolic intermediates and acclimation period (if required to initiate metabolism) can be used to characterize the relative potential a substrate may possess for interference with the utilization of another substrate when they are present as mixed carbon source. However, growth rates provide a better basis than the accumulation of intermediates for judgment of relative interference potential between two compounds when no acclimation period is needed to initiate metabolism of either compound.

4. In general the order of substrate removal in the combined system was consistent with the potential sequence predicted from the controls. This suggests that, in general, the potential sequence observed in the controls can be used for predicting the order of substrate removal in the combined system.

5. In the continuous flow systems, the order of priority of substrate removal was consistent throughout the experiment regardless of various changes in microbial predominance which resulted from shifting the dilution rate.

6. In the continuous flow systems a significant amount of metabolites such as acetate were found at moderately high and high dilution rates. The excretion of organic metabolites was accompanied, usually, by the presence in the effluent of one or more of the substrates in the inflowing feed.

7. Heavy wall growth and the presence of flocculated cells were observed at high dilution rate. This occurrence hampered accurate sampling and analysis at these dilution rates.

8. The maximum specific growth rate on a multicomponent substrate estimated from the batch systems is generally smaller than that observed in continuous flow systems.

9. In general at least ten detention times will be required for a chemostat system to approach a new "steady-state" condition after a change in dilution rate.

10. In applying "chemostat" studies to the design of an activated sludge system, one should base values on averages taken over extended periods of time rather than one or two values obtained from a "steady-state" condition.

CHAPTER VII

SUGGESTIONS FOR FUTURE WORK

The following future work would be of interest:

1. Studies on the utilization of two or more carbon sources in continuous flow systems using known mixture of microorganisms (i.e., mixed pure cultures).

2. Studies on mixtures of normally readily-available carbon source, e.g., carbohydrate, with compounds which are rather resistant to metabolism or which exert rather severe selective pressure on the microbial population, e.g., phenol.

3. Studies on the use of multistage continuous flow systems with a combined feed of one preferred carbon source (e.g., glucose or sucrose) and one or two poor carbon sources (e.g., phenol and butyric acid).

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APPENDIX

SYSTEM PARAMETERS FOR THE STUDY OF MULTICOMPONENT SUBSTRATE SYSTEMS

1	2	3	4	5	6	7	8	9	10
System	Figure No.	Cell History	Unit	Growth Rate $\mu(hr^{-1})$	COD Removal Rate $Kt(hr^{-1})$	Substrate Removal Rate $Ks(hr^{-1})$ or mg/l	$\frac{\mu}{Kt}$ (%)	Yield (%)	Yield (%)
	3a	Glucose Acclimation	Galactose Control	0.171	0.149	0.163	114.8	52.0	46.7
	3b		Fructose Control	0.192	0.195	0.200	97.9	50.0	50.2
	3c		Glucose Control	0.241	0.250	0.274	96.4	55.0	52.6
Glucose-Galactose-Fructose	4		Combined	0.239	0.283	Kglu=0.281, Kf=0.262, Kgal=0.428	84.5	45.0	52.2
	5a	Galactose Acclimation	Glucose Control	0.348	0.347	0.362	100.3	41.7	47.6
	5b		Fructose Control	0.269	0.304	0.302	89.2	50.0	50.5
	5c		Galactose Control	0.216	0.217	0.253	99.5	50.0	50.3
	6		Combined	0.347	0.353	Kglu=0.358, Kf=0.365, Kgal=0.618	98.2	48.0	42.2
	7a	Fructose Acclimation	Galactose Control	0.232	0.277	0.278	83.8	62.8	58.6
	7b		Glucose Control	0.319	0.389	0.412	82.0	50.0	43.8
	7c		Fructose Control	0.302	0.325	0.365	92.9	49.2	41.6
	8		Combined	μ_1=0.350 μ_2=0.172	Kt_1=0.324 Kt_2=0.143	Kglu=0.408, *Kf=88.0, Kgal=0.690	108 120	52.5	50.6
	11a	Glucose Acclimation	Sorbitol Control	0.221	0.283	0.280	78.2	73.2	66.0
	11b		Ribose Control	0.124	0.144	0.139	86.2	53.3	64.9
	11c		Glucose Control	0.261	0.300	0.332	87.0	61.1	59.4
Glucose-Ribose-Sorbitol	12		Combined	μ_1=0.272 μ_2 0.010	Kt_1=0.250 Kt_2=0.013	Kg=0.362, Ks= 0.56 *Kr=44.0	108.7 77	56.7	65.1
	13a	Soribtol Acclimation	Ribose Control	0.128	0.179	0.195	71.5	48.6	51.6
	13b		Glucose Control	0.264	0.316	0.318	83.6	62.4	56.6
	13c		Sorbitol Control	0.278	0.340	0.345	81.8	60.0	54.7
	14		Combined	μ_1=0.312 *μ_2, zero order μ_3=0.012	Kt_1=0.368 Kt_2=0.016	Kg=0.364, Kr=27.8 *Ks_1= 14.1, *Ks_2=127 *Ks_3=4	84.8 75.1	50.0	62.9
	15a	Ribose Acclimation	Glucose Control	0.245	0.270	0.271	90.8	53.3	53.9
	15b		Sorbitol Control	0.174	0.181	0.192	96.1	50.0	51.8
	15c		Ribose Control	0.129	0.143	0.144	90.3	55.0	47.5
	16		Combined	μ_1=0.246 μ_2=0.089	Kt_1=0.274 Kt_2=0.129	Kglu=0.308, Kr=0.372 Ks=0.908	89.8 60.0	51.6	53.6

*Denotes linear removal rate.

APPENDIX (Continued)

1	2	3	4	5	6	7	8	9	10
System	Figure No.	Cell History	Unit	Growth Rate $\mu(hr^{-1})$	COD Removal Rate $Kt(hr^{-1})$	Substrate Removal Rate $Ks(hr^{-1})$ or mg/l	$\frac{\mu}{Kt}$ (%)	Yield (%)	Yield (%)
	19a	Glucose Accli-mation	Glucose Control	0.306	0.298	0.328	102.7	35.0	45.6
	19b		Galactose Control	0.197	0.200	0.217	98.5	43.4	42.5
	19c		Sucrose Control	0.344	0.315	0.292	109.0	60.0	57.6
	20		Combined	0.355	0.340	*Ks=11.0 Kglu=0.253, Ks=0.246 *Kgal=5.5 and 100.0	104.5	40.0	49.0
Glucose-Galactose-Sucrose	21a	Galactose Accli-mation	Glucose Control	0.264	0.322	*90.0	82.0	50.0	51.1
	21b		Galactose Control	0.189	0.278	0.302	68.0	46.2	49.4
	21c		Sucrose Control	0.258	0.354	3.68	72.8	50.0	53.8
	22		Combined	0.280	0.385	*Kglu=104.0, Ks=0.243 *$Kgal_1$=21.0,*$Kgal_2$=0.694	72.7	46.1	58.9
	23a	Sucrose Accli-mation	Glucose Control	0.406	0.422	0.426	96.2	58.3	60.0
	23b		Galactose Control	0.344	0.357	0.353	96.3	47.6	52.7
	23c		Sucrose Control	0.404	0.414	0.443	97.7	57.1	55.3
	24		Combined	0.394	0.382	Kglu=0.346, Ks=0.451 *$Kgal_1$=10.5, $Kgal_2$=0.406	103.1	50.0	48.4
	27a	Glucose Accli-mation	Sucrose Control	0.216	0.220	0.228	98.2	57.1	51.4
	27b		Xylose Control	0.086	0.115	0.135	74.9	55.0	43.9
	27c		Glucose Control	0.287	0.288	0.351	99.7	46.7	50.8
	28		Combined	μ_1=0.270 μ_2=0.005	Kt_1=0.330 Kt_2=0.024	Kg=0.328, Ks=0.214 *Kx=50.0	82.0 21.3	53.4	47.1
Glucose-Xylose-Surcrose	29a	Xylose Accli-mation	Sucrose Control	0.314	0.552	0.492	56.9	56.7	56.5
	29b		Xylose Control	0.195	0.243	0.246	80.4	48.1	47.8
	29c		Glucose Control	0.278	0.385	0.405	72.3	65.0	50.1
	30		Combined	0.294	0.497	Kg=0.400, Ks=0.334 Kx=0.405	59.2	55.0	57.8
	31a	Sucrose Accli-mation	Sucrose Control	0.265	0.278	0.280	95.4	45.0	45.2
	31b		Xylose Control	0.103	0.104	0.104	99.0	42.0	41.7
	31c		Glucose Control	0.260	0.273	0.286	95.3	42.5	45.1
	32		Combined	μ_1=0.235 μ_2=0.013	Kt_1=0.246 Kt_2=0.02	Kg=0.333, Ks=0.214 Kx=0.223	95.6 65.0	41.7	42.6

*Denotes linear removal rate

APPENDIX (Continued)

1	2	3	4	5	6	7	8	9	10
System	Figure No.	Cell History	Unit	Growth Rate $\mu(hr^{-1})$	COD Removal Rate $Kt(hr^{-1})$	Substrate Removal Rate $Ks(hr^{-1})$ or mg/l	$\frac{\mu}{Kt}$ (%)	Yield (%)	Yield (%)
Glucose-Ribose-Glycerol	35a	Glucose Acclimation	Glucose Control	0.350	0.346	*103.0	101.1	50.0	52.8
	35b		Ribose Control	0.238	0.330	0.334	0.334	72.1	68.5
	35c		Glycerol Control	0.324	0.345	0.345	93.9	53.3	51.4
	36		Combined	0.378	0.382	Kglu=0.423, Kr=0.605 Kgly=0.710	98.9	54.3	51.4
	37a	Ribose Acclimation	Glucose Control	0.359	0.393	0.406	91.3	64.6	58.0
	37b		Ribose Control	0.255	0.264	0.263	96.6	56.7	57.4
	37c		Glycerol Control	0.290	0.293	0.294	98.9	52.7	55.3
	38		Combined	0.345	0.338	Kglu=0.358, Kr=0.394 *Kgly=125.0	102.0	45.7	61.1
	39a	Glycerol Acclimation	Glucose Control	0.306	0.322	0.320	95.0	50.0	50.9
	39b		Ribose Control	0.150	0.170	0.175	88.3	56.0	56.7
	39c		Glycerol Control	0.247	0.262	0.264	94.3	46.7	50.1
	40		Combined	0.239	0.226	Kglu=0.312, Kr=0.440 *Kgly=96.0	105.8	45.0	51.8
Glucose-Sucrose-Glycerol	43a	Glucose Acclimation	Glucose Control	0.372	0.421	0.428	88.4	60.0	60.8
	43b		Sucrose Control	0.304	0.347	0.362	87.7	58.4	58.4
	43c		Glycerol Control	0.216	0.266	0.278	81.2	55.0	59.88
	44		Combined	μ_1=0.345 μ_2=0.069	K_1=0.394 K_2=0.080	Kglu=0.396,Ks=0.294 Kgly=0.495	92.2 86.3	52.5	55.4
	45a	Sucrose Acclimation	Glucose Control	0.289	0.390	0.392	73.6	62.0	58.6
	45b		Sucrose Control	0.248	0.344	0.330	72.2	58.3	56.2
	45c		Glycerol Control	0.192	0.251	0.251	76.5	54.3	48.6
	46		Combined	μ_1=0.280 μ_2=0.046	K_1=0.305 K_2=0.092	Kglu=0.380,Kgly=0.632 *Ks_1=15.8,*Ks_2=115	91.8 50.0	45.0	45.4
	47a	Glycerol Acclimation	Sucrose Control	0.313	0.352	0.386	88.9	78.3	78.1
	47b		Glucose Control	0.344	0.399	0.431	86.2	68.1	64.6
	47c		Glycerol Control	0.294	0.354	0.355	83.1	71.4	58.0
	48		Combined	0.390	0.430	Kglu=0.496,Kgly=0.415 *Ks_1=8.0, *Ks_2=156	90.7	62.2	64.5

*Denotes linear removal rate

APPENDIX (Continued)

1	2	3	4	5	6	7	8	9	10
System	Figure No.	Cell History	Unit	Growth Rate $\mu(hr^{-1})$	COD Removal Rate $Kt(hr^{-1})$	Substrate Removal Rate $Ks(hr^{-1})$ or mg/l	$\frac{\mu}{Kt}$ (%)	Yield	Yield
	53a	Glucose Acclimation	Glucose Control	0.309	0.319	0.325	96.8	60.0	56.6
	53b		Sorbitol Control	0.212	0.275	0.270	77.1	57.5	57.6
	53c		Galactose Control	0.150	0.172	0.214	87.3	62.8	58.6
	53d	Xylose Acclimation	Xylose Control	0.129	0.163	0.190	79.1	78.6	69.5
	54		Combined	0.293	0.326	Kglu=0.426, Kgal=0.432 Kx=0.387, Ks=0.272	89.9	63.3	62.3
	55a		Xylose Control	0.233	0.264	0.278	88.3	51.1	50.9
	55b		Glucose Control	0.249	0.323	0.358	77.2	58.0	58.1
	55c		Sorbitol Control	0.225	0.255	0.258	88.2	54.0	53.4
	55d		Galactose Control	0.216	0.254	0.260	85.1	60.0	60.2
	56		Combined	0.243	0.301	Kglu=0.266,*Kx=43.4 Kgal=0.228,*Ks_1=46.1 *Ks_2=7.2	80.8	60.0	62.3
Glucose-Galactose-Xylose-Sorbitol	57a	Galactose Acclimation	Glucose Control	0.261	0.332	0.340	78.7	61.8	63.4
	57b		Galactose Control	0.187	0.276	0.278	67.8	65.0	62.2
	57c		Sorbitol Control	0.218	0.336	0.350	64.9	62.9	59.1
	57d		Xylose Control	0.092	0.137	0.143	67.2	44.0	40.2
	58		Combined	0.242	0.248	Kglu=0.360, Kx=0.399 Kgal=0.315, Ks=0.163	97.6	66.7	73.7
	59a	Sorbitol Acclimation	Glucose Control	0.277	0.322	0.469	86.1	57.1	56.2
	59b		Sorbitol Control	0.272	0.278	0.298	97.9	57.5	56.1
	59c		Galactose Control	0.210	0.272	0.280	77.2	56.7	59.4
	59d		Xylose Control	0.121	0.143	0.139	84.6	60.0	49.6
	60		Combined	0.270	0.299	Kglu=0.254,*Kx=58.0 Kgal=0.300, Ks=0.06	90.1	36.7	59.9

*Denotes linear removal rate

Explanation of Columns in Appendix

1. Column 1 shows the substrate combination used in this study.
2. Column 2 shows the figure number shown in the text of this thesis.
3. Column 3 shows the acclimated cells used.
4. Column 4 shows the substrate employed in each system.
5. Column 5 shows the specific rate of growth during the log phase, $\mu(hr^{-1})$.
6. Column 6 shows the specific rate of total COD removal during the log phase, $Kt(hr^{-1})$.
7. Column 7 shows the specific rate of substrate COD removal during the log phase $Ks(hr^{-1})$.
8. Column 8 shows the ratio of μ/kt (%); i.e., column 5/column 6.
9. Column 9 shows the sludge yield in percent calculated as the slope of the curve for solids accumulation versus total COD removal.
10. Column 10 shows the sludge yield in percent calculated as increase in biological solids divided by total COD removal.

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